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THE STUDY OF PROBLEMS OF IMMUNITY BY THE TISSUE CULTURE METHOD

III. A METHOD FOR DETERMINING THE RESISTANCE OF INDIVIDUALS TO DIPHTHERIA INFECTION

MONTROSE T. BURROWS AND YOSHIO SUZUKI

The Department of Pathology, Washington University Medical School, St. Louis, Missouri

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Many attempts have been made to devise a simple and accurate laboratory method for determining the presence of toxic and antitoxic substances in the blood of persons suffering from acute infections of various kinds and in the blood of normal individuals. Several methods adapted for the study of certain toxins have resulted. Many of these in the hands of most workers have been difficult to manipulate. Others have failed to give the qualitative and quantitative data necessary. It has been evident since the beginning of the study of toxins and antitoxins that their presence or absence must be determined by testing their effect upon animal tissues. Other and more accurate methods will probably not be devised until the chemical and physical properties of these substances are better understood.

For a long time it has seemed apparent that the tissue culture method might aid very materially in making such determinations. Stimulated by the urgent need of a more careful study of many of the cases of diphtheria and other infections as they have been seen in the hospital and as they are reported from army camps we undertook recently to apply the method in this direction.

As is well known the tissue cells of many animals grow readily not only in their own plasma but also in drops of plasma of many other animals. It seemed evident, therefore, that the tissue of an

animal particularly susceptible to a given toxin might be used to detect the presence of this toxin or substances which neutralize this toxin in the plasma of any animal in which the tissue will grow.

In a previous article (1) we have already shown how this method may be thus directly applied for the study of natural immunity. The object of the experiments was to determine whether the natural immunity of certain animals is the result of a specific resistance on the part of the cells, whether it is due to the neutralizing substances in the plasma of the animals, or, both. Chickens and guinea-pigs are susceptible to diphtheria toxin. Rats are resistant. The cells of the rats grow readily, not only in their own plasma but in the plasmata of chickens and guinea-pigs. In turn, tissue cells of either chickens or guinea-pigs grow actively in any one of the plasmata of the three animals. We tested the resistance of the cells of each of these animals to diphtheria toxin in the plasma of each of the animals. Through these experiments it was possible to show that the natural immunity of rats for diphtheria toxin is due both to a specific resistance of their cells and to the existence of neutralizing substances in their blood which protect, also, the cells of other less resistant animal tissues. A similar study was likewise made with tetanus toxin.

In another paper (2) we have also shown how the method may be applied for the standardization of antitoxin and the study of passive immunity. For the standardization of diphtheria antitoxin the sensitive chicken cells were grown in the plasma of normal chickens to which mixtures of toxin and antitoxin were added. Chickens were used for the study of passive immunity. It was shown that the passive immunity of a chicken previously injected with diphtheria antitoxin could be determined by cultivating fragments of the heart muscle of chick-embryos in drops of the plasma of the immunized chicken to which various quantities of diphtheria toxin had been added.

Chicken tissue can be readily cultivated in drops of human plasma. It became evident, therefore, that one might use this tissue to develop a method for the detection of small quanti-

ties of diphtheria toxin and antitoxic substances in the blood of patients or normal individuals. As an introduction to this particular use of the method we have studied the neutralizing value of the blood of a number of normal adults and children.

THE METHOD

Fragments of the ventricular muscle of the heart of fifteen and sixteen day chick-embryo, carefully cut so that they are approximately 1 mm. in diameter, are placed on the surface of a specially cleaned cover glass and covered with a layer of medium consisting of 1 part of the plasma to be tested and 1 part of a 0.9 per cent NaCl solution (control) or 1 part of the plasma to be tested and 1 part of any one of various dilutions of diphtheria toxin in 0.9 per cent NaCl solution. The medium is spread about the tissue so that it forms a layer approximately 0.5 mm. in thickness. A hollow ground slide previously rimmed with vaseline is then immediately inverted over the cover glass so that the tissue and the medium become sealed within the hollow chamber. When the plasma has clotted the slide is inverted, the cover is further sealed with paraffin and the slide is incubated at a temperature suitable for the chicken tissue, 39.4°C.

The toxin used for all these experiments was obtained from Parke, Davis and Company. The strength of this toxin, as indicated on the label is: L + Dose = 0.29.

For the study of each sample of blood at least 18 cultures have been prepared and in making these at least 8 different dilutions of the toxin have been tested. In many of the experiments we have studied as many as 12 different dilutions of toxin. Since chicken tissue can be made to grow in 100 per cent of the drops of the control medium it has been found unnecessary to prepare more than 2 cultures of each toxin dilution. The general procedure used in making the test is illustrated in table 1—Plasma no. 14 of the table below (table 4).

Fresh toxin dilutions have not been made for each experiment. Fresh dilutions have been made as a routine every twenty-four or forty-eight hours. The diluted toxin is left in the ice-box except during the time when samples are taken from it.

The toxin dilutions have been made by the drop method and by direct measurement. The latter has been more satisfactory. They are made in ordinary specially cleaned small glass test tubes. The highest concentration of toxin which we have used has been 10 per

cent. Higher concentration may, however, be used without injuring the growth of the cells as long as the isotonicity of the solution is not too greatly disturbed and the plasma neutralizes the action of the toxin.

TABLE 1

Medium: Plasma of case 14, (table IV), 1 part; various dilutions of diphtheria toxin, 1 part
Control: The same plasma, 1 part; 0.9 per cent NaCl solution, 1 part
Tissue: Fragments of the ventricular muscle of the heart of a fifteen-day chick-embryo

TOXIN, NUMBER OF TIMES DILUTED	CULTURE NUMBER	GROWTH AFTER		
		24 hours	48 hours	72 hours
10	1	+	+++	++++
	2	+	+++	++++
50	3	+	+++	++++
	4	+	+++	++++
100	5	+	+++	++++
	6	+	+++	++++
500	7	+	+++	+++
	8	+	+++	++++
1000	9	+	+++	++++
	10	+	++	+++
3000	11	+	+++	++++
	12	+	+++	++++
5000	13	+	+++	++++
	14	+	+++	++++
7000	15	+	+++	++++
	16	+	+++	++++
Control	17	+	+++	++++
	18	+	+++	++++

From the 10 per cent solution of toxin first prepared the next lower concentration is made by carefully measuring known quantities of it into a known quantity of 0.9 per cent NaCl solution and so on.

The cultures are prepared as soon as the plasma is obtained and before any possible clotting has taken place.

The preparation of plasma. Some difficulty may arise in preparing human plasma unless certain precautions in technique are especially taken. In the preparation of plasma from lower animals it has been customary here in the laboratory to draw the blood from an artery through a carefully cleaned and oiled cannula into specially cleaned glass test-tubes. A rather slender long test-tube is used. This test tube is filled half full of blood and as soon as filled it is corked and plunged into an ice water, or ice, salt and water bath so that it becomes chilled at once. Care must be taken when salt is used not to freeze the plasma. As soon as this blood is chilled it is centrifugalized by plunging the test-tubes containing the blood into large centrifuge recepticles (50 cc. capacity) filled with ice, salt and water. A rapidly running electric centrifuge (International Equipment Company, size 1, style A) is suitable for this work.

For preparing human plasma we have used glass test-tubes similar to those used in animal experiments. They are 12 cm. long and have an inside diameter of 7 mm. They are fitted with a large cork into which a hole has been cut so that the cork fits over the end of the tube. These test-tubes are made in the laboratory out of ordinary soft glass tubing having a thick wall. They are specially cleaned to prevent chemical contamination of the medium.

A cannula cannot be used in obtaining blood from man. We use a 10 cc. Luer Syringe fitted with a needle, gauge no. 19, the point of which is kept very sharp. In many cases these syringes have been carefully cleaned, coated with olive oil and sterilized in the autoclave. The needles have been similarly cleaned, coated and sterilized. A simple procedure is to place such clean needles in small test-tubes fitted with cotton stoppers. The needles are placed point down in the tube. The sharp point is held from the bottom of the tube by a wire cleaning rod having a loop end. The syringe with two such needles in test-tubes are wrapped in gauze and canvas and sterilized.

For most of this work we have simply boiled the needle and syringe in water rinsing them later in sterile isotonic NaCl solution to prevent any haemolysis. In cold weather an ice water bath is used to chill the blood. In hot weather a little salt is added to the bath. A small flask with this is taken to the ward or place where blood is obtained. The blood must be chilled as soon as taken.

For the preparation of serum it is only essential to obtain the blood. For the preparation of plasma it is essential that the blood be taken as free as possible from tissue contamination and that it be agitated as

little as possible. Chicken plasma taken free from tissue contamination may be handled roughly. This is not true of human and mammalian plasmata. Roughly handled blood will invariably clot and prevent the test. The needle must be plunged directly into the vein at the first thrust; otherwise a new needle must be taken. The blood must be allowed to flow into the syringe. Sucking the side wall of the vein into the mouth of the needle often causes clotting to take place before the plasma can be obtained and used. In obtaining plasma for these tests we have used a tourniquet, and have cleaned the skin with 80 per cent alcohol. We take about 5 cc. of blood, transfer it immediately to the tubes, allowing it to run in gently, chill, centrifuge within five or ten minutes and use it, if possible, within a half hour or an hour after it is taken. Many samples of human plasma kept cold will remain unclotted for several days. Any sample of human plasma prepared with care will remain unclotted for an hour or often longer.

In cold weather we allow the centrifuge to run for two or even three minutes. In hot weather two minutes is as long as any blood is centrifugalized. The ice in the cup is melted within this time and the plasma is warm. As soon as the tubes are removed from the centrifuge they are again plunged into the ice water. From this time on the plasma is kept cold until it is placed on the cover glass.

The preparation of the culture media. The medium for the culture is prepared in slender tubes. We have used tubes $3\frac{1}{2}$ inches long and $\frac{3}{16}$ inch in diameter. These are placed in a flask containing ice and water. A pipet is placed in each tube. These pipets have long slender ends which reach the bottom of the tube; they are made of ordinary glass tubing and are fitted with a rubber bulb, which has been cleaned carefully. Each pipet has a file mark at the neck of the slender end. Plasma is first added to the tubes. The pipet is filled with plasma up to the file mark and emptied into the bottom of the tube. With the same pipet an equal quantity of NaCl solution 0.9 per cent or diluted toxin is taken and thoroughly mixed with the plasma. Since very small quantities of medium are necessary in preparing the cultures we use very small quantities of plasma and diluting agent.

The cleaning of glassware. On account of the small quantity of medium used in preparing tissue cultures and the large amount of glass surfaces to which it is exposed at different times it is very essential that great care be taken in cleaning the glassware if constant results are to be expected. We have used the method that was introduced in the laboratory by one of us a few years ago. All the glass-

ware is treated alike, that which has been previously used as well as the new. It is boiled first in soap and water, rinsed in tap water and placed in a weak sulphuric acid solution for several hours. When removed from this last solution it is again rinsed in tap water and placed in a dish of distilled water. The final cleansing is made with steam. A jet of hot steam is allowed to play for several minutes on the inside of each tube, pipet, flask and dish. The apparatus for steaming the glassware is similar to that described by Finlay (3). An ordinary flask, Erlenmeyer or otherwise, is fitted with a tight cork through which passes a slender glass delivery tube. A funnel is placed over this tube in such a way that the tube passes through the stem of the funnel. The stem of the funnel, which is cut very short, is fitted tightly into the outer part of the cork. By this procedure the funnel is held firmly in place and its lower end is closed. It acts as a receptacle for the condensed steam. The flasks, pipets, dishes and tubes are inverted over the delivery tube through which the steam passes. We use distilled water in the flask.

After steaming, the glassware is dried and sterilized at 200°C for one hour.

The prevention of infection of the cultures with bacteria from the air. During the preparation of the cultures they are exposed for some time to the outside air and in an ordinary room a large number will invariably become contaminated with bacteria. The cultures must be kept sterile at all times. If a small room free from dust and draughts is not available a box may be used. The one which we have used is 3 feet wide, 14 inches high and 2 feet deep. The sides are made of wood. The front, top and back are fitted with glass windows. The back reaches only to within 4 inches of the table, leaving an opening for inserting the hands and material into the box. The bottom is open. The box is placed on a black top table or a table covered with a black cloth. All cultures have been prepared in such a box. This box has been used by one of us for this purpose for several years and in many different rooms as well as in the open. It has always been found practical.

The control. In making tests of this kind it is most essential that the growth of cells in the cultures is one that can be predicted and regulated. Many tests of different substances previously made with the tissue culture have been found later to be of little value on account of the failure to have had a proper control. The finding of methods for controlling growth in the tissue culture formed one of the

early problems of one of us in the study of this method. To detect peculiarities of plasma and the presence of toxic substances within a plasma one must be certain of what is to be expected under normal conditions.

A statement of the theoretical deductions and facts elucidated by the development of a method for controlling growth of certain tissues in culture may aid in the understanding and the application of the more minute details of the technique of preparing the cultures.

The tissue culture cannot be compared in detail with the bacterial culture. The tissue cells planted in plasma do not grow at the expense of the plasma. Single cells may show movement in this medium but they do not grow. After a short period of movement they show evidence of exhaustion. Growth takes place only about fragments of tissue. When fragments of 1 mm. or less in diameter are used the growth is inversely proportional to the size of the fragment. This proportion holds for a given tissue only when the fragments are of equal cellular content and arrangement. A small fragment may be made large by teasing the cells apart. The growth from such a fragment is less than that from one of the same size which is compact.

The growth of cells in a tissue culture is the growth of the cells on the periphery of the fragment or cells that have migrated there. The nutrient material for the growth of these cells comes from the cells disintegrating within the fragment. The growth as it is observed in the culture is not materially different from movement or cell migration. Both are none other than a simple transfer of materials from the fragment to the media without (4). The maximum growth or migration of cells from the fragments of most tissues is seen about fragments of that tissue 1 mm. in diameter. The interchange or diffusion of substances is disturbed in fragments of greater thickness. The growth may be the same but it is never greater about larger fragments (5).

Any thing that again disturbs the diffusion of material from the fragments into the outer medium disturbs the growth and

movement of cells. Again, oxygen does not diffuse readily into the plasma to a depth greater than 0.7 mm. In preparing the cultures it is essential that the cover glass surface and the thickness of the medium layer be kept constant. Another factor is the density of the clot. Dilution of the plasma with liquids leads to a greater growth of cells (6). In all cases we have diluted the control medium exactly as in the experiment using 0.9 per cent NaCl solution. The layer of medium in each culture has been made approximately 0.5 mm. in thickness. Slight variations make very little difference. With a little practice one can obtain a suitably thick layer without difficulty. Approximately 0.5 mm. is sufficiently accurate.

The cleaning of cover glasses. A cover glass surface over which the medium does not spread easily is essential. All the cover glasses both new and those previously used are cleaned first by boiling in soap and water. They are then boiled three or four times in distilled water and placed in absolute alcohol for at least twenty-four hours. From this they are taken, dried and polished with an old silk or linen cloth, placed in Petri dishes and sterilized dry at 160°C. for one hour. If the sterilizer is hotter than this the surface is often changed and the drops of plasma tend to spread over them. Cover glasses cleaned in the manner just described have shown a uniform kind of surface. The drops of plasma hold up well on their surfaces.

The choice of a tissue. The cells that grow most actively in the tissue culture are the connective tissue cells. Very active growth is, however, rarely seen about fragments of pure connective tissue such as subcutaneous tissue. The active growths of these cells are seen about fragments of glands or skin or from other tissue containing epithelial cells. The epithelial cells disintegrate at the expense of the connective tissue and supply the nutriment. About these fragments the growth of the connective tissue is often excessive—far in excess of the original amount present. From fragments of pure connective tissue, for example, subcutaneous tissue, little more than a movement of cells is generally seen.

Great difficulty has always been encountered, however, in controlling the growth of cells from fragments containing epithelium.

The epithelium may grow or show movement and when it does it inhibits completely the growth of the connective tissue. Again, when it lies near the edge of the fragment the medium frequently undergoes liquefaction or retraction and no growth of cells results.

From fragments of the more differentiated connective tissue the growth is often move active than from subcutaneous tissue. Such is the case of heart muscle. Liquefaction of the medium does not take place about these fragments. A large number of fragments of equal cellular content may be easily obtained from the ventricle of the chick-embryo. The tissue is firm and can be cut without disturbing the cellular density and arrangement in the fragment. Moreover, it grows readily and the extent and type of the growth can be predicted if care is taken in the preparation of the cultures.

The heart may be obtained easily in a sterile condition from the embryo. We open the eggs with sterile scissors, remove the embryo to a sterile dish, take out the heart with clean sterile instruments and place it in a sterile Petri dish. It is then cut into fragments with a sharp scalpel of large size. The cutting is done against the bottom of the dish or another clean and sterile piece of glass. By this means cleanly cut and solid fragments can always be obtained. As soon as cut they are transferred on the point of the knife or a pair of forceps to the cover glass, covered with plasma and sealed in the hollow slide chamber. Slight drying will have little effect. It is best to work rapidly, however. The tissue may be kept moist with isotonic salt solution. This is not advisable when quantitative results are expected because in such small drops the salt solution clinging to the instruments and tissue causes a noticeable dilution.

The age of the embryonic tissue. In the previous papers we have noted that the tissue cells of the younger embryos grow for a time in a greater amount of diphtheria toxin than those of older ones. The heart muscle cells of younger embryos may grow for a time in a concentration of toxin as great as 1/500 while those of older ones or of foetuses will not grow in a concentration greater than 1/6000.

It is most suitable to use, however, a tissue which grows rapidly and actively. Embryonic and foetal tissues grow more rapidly and actively than those of young animals and adults.

TABLE 2

Medium: Plasma of case 5, (table IV), 1 part; various dilutions of diphtheria toxin, 1 part
Control: The same plasma, 1 part; 0.9 per cent NaCl solution, 1 part
Tissue: Fragments of the ventricular muscle of the heart of fifteen-day chick-embryo

TOXIN, NUMBER OF TIMES DILUTED	CULTURE NUMBER	GROWTH AFTER		
		24 hours	48 hours	72 hours
10	1	−	−	−
	2	−	−	−
50	3	−	−	−
	4	−	−	−
100	5	−	−	−
	6	−	−	−
500	7	−	−	−
	8	−	−	−
1000	9	±	−	−
	10	±	−	−
3000	11	±	±	+
	12	±	±	+
5000	13	±	±	+
	14	+	++	+++
7000	15	+	++	+++
	16	+	++	+++
Control	17	+	+++	++++
	18	+	+++	++++

Again, when the younger embryonic tissue is used a sharp line of demarcation does not exist where one could say the medium is toxic or that the toxin is sufficiently neutralized. The cells from the fragments of the heart muscle of fifteen or sixteen day chick embryos grow readily and actively after twelve or twenty-four hours. They are sensitive in chicken plasma to a concen-

tration higher than 1/6000 of the diphtheria toxin which we have used. The line of demarcation is sharp in all of our series. In many of the tests one finds the cells growing actively in one

TABLE 3

Medium: Plasma of case 10 (table 4), 1 part; various dilutions of diphtheria toxin, 1 part
Control: The same plasma, 1 part; 0.9 per cent NaCl solution, 1 part
Tissue: Fragments of the ventricular muscle of the heart of a fifteen-day chick-embryo

TOXIN, NUMBER OF TIMES DILUTED	CULTURE NUMBER	GROWTH AFTER		
		24 hours	48 hours	72 hours
10	1	−	−	−
	2	−	−	−
50	3	+	++	+++
	4	+	++	+++
100	5	+	+++	++++
	6	+	+++	++++
500	7	+	+++	++++
	8	+	+++	++++
1000	9	+	+++	++++
	10	+	+++	++++
3000	11	+	+++	++++
	12	+	+++	++++
5000	13	+	+++	++++
	14	+	+++	++++
7000	15	+	+++	++++
	16	+	+++	++++
Control	17	+	+++	++++
	18	+	+++	++++

dilution, no growth being seen in any of the media having a greater concentration of the toxin; table 2. In another there may be a small growth of cells in the higher concentration of toxin during the first twenty-four hours. These cells soon disintegrate or the growth remains very small in amount and a sharp point is established; table 3.

In all of the tables the relative amount of growth is indicated by the number of + marks. When no growth takes place the mark − is used. The sign ± indicates the growth of a few cells. All cultures were followed for seventy-two hours. Readings were recorded every twenty-four hours.

The concentration of toxin indicated in the tables is the concentration in the salt solution. The actual amount in the medium is one-half that amount.

EXPERIMENTS AND DISCUSSION

The blood of 27 adults and 11 children have been studied by this method. The results of the study are given in tables 4 and 5. In most instances the Schick reaction was also performed The relative value of the Schick test as compared with our method is indicated in these tables.

In tables 4 and 5 the black lines cover the dilution in which growth takes place rapidly in the cultures. In this series there are several individuals that had had diphtheria at a previous time. There are also two carriers. A few of those having had the disease; cases 10, 13, 14, 19, 26 and 27 (table IV), show a definite resistance. The carriers, also, are resistant. Individuals, who have had diphtheria, do not however, necessarily show a high resistance; case 23 (table IV).

In table 5 it is noted that the children show practically the same variations as those of adults. After two months in a number of instances the blood of the same individuals studied in table IV was studied again by one of us. A new toxin was used but one having the same tested strength of the other. In a few of the cases slight differences were noted in the results. In others no such differences were seen. It is known that the plasma of an individual may be more suitable as a medium at one time than another. Plasma taken from animals that have been starved for twenty-four or forty-eight hours is invariably better than that taken from a feeding animal. Plasma taken from animals fed on a protein diet has been found to be a better medium than that taken from an animal fed on carbo-

TABLE NUMBER 4.

No	Sex	Age in years	Previous Attack of Diptheria	Schick Test	Toxin Dilutions							
					7000	5000	3000	1000	500	100	50	10
5	M	33	none	+								
7	M	29	none	+								
8	M	22	none	+								
15	M	29	none	+								
22	M	21	none	+								
18	M	50	none	+								
21	M	24	none	+								
20	F	20	none	+								
23	M	21	5 years ago**	+								
1	M	35	none	+								
6	M	23	about 16** years ago	−								
12	M	57	none	−								
3	M	25	none	−								
11	M	26	none	−								
2	M	17	none	−								
10	M	22	8 years ago **	−								
17	M	23	21 years ago**	−								
24	M	22	none	−								
25	M	26	none	−								
4	M	35	none	−								
9	M	52	none	−								
13	M	37	35 years ago	−								
14	F	23	15 days* ago **	−								
16	M	37	none	−								
19	M	23	14 years ago **	−								
26	F	16	17 days* ago **	−								
27	F	30	25 years ago									

** Treated with antitoxin * Diphtheria carrier

hydrates or fats. The differences are not striking, however. A plasma taken after a full meal is cloudy with fat. Growth is generally inhibited to a noticeable extent in such a plasma (9). To what extent variation in climate, food, etc., may effect slight differences in the relative resistance of tissues in the cultures is a problem of interest but one which has not been studied by us up to the present time.

TABLE NUMBER 5.

No.	Age	Sex	Previous Attack of Diphtheria	Schick Test	Toxin Dilutions							
					7000	5000	3000	1000	500	100	50	10
4	14 mo.	M	none									
7	13 mo.	F	none	+								
2	11 mo.	M	none	+								
5	21 mo.	M	none	+								
9	20 mo.	M	none	+								
3	10 mo.	F	none									
1	4 mo.	M	none									
8	18 mo.	M	none	−								
10	10 yrs.	M	immunizing dose									
6	13 mo.	M	none									
11	11 yrs.	M	immunizing dose	−								

Several years ago Marks showed that diphtheria antitoxin standardization may be made by injecting mixtures of toxin and antitoxin into the subcutaneous tissue of guinea pigs. Toxin injected alone causes local oedema. This is prevented by adding sufficient quantities of antitoxin to the toxin solution. Marks claimed that this method is more sensitive than that used by Ehrlich and others for such standardization.

Römer noted that necrosis followed the intracutaneous injection of diphtheria toxin in guinea pigs. He introduced a

method of diphtheria antitoxin standardization based upon this fact. If necrosis follows the injection of toxin and antitoxin, free toxin must be present in the mixture. Completely neutralized mixtures cause no necrosis.

This method according to Zinsser has not been found easy except in the hands of those that have had a great amount of practice and experience with it. The method has been used, however, by several investigators for the detection of toxin in the blood of diphtheria patients (7).

The generally used method for determining the resistance of individuals to diphtheria is that introduced by Michiels and Schick. They made their injections directly into the skin of the patient. This method has been used in many of the large epidemics for separating those individuals that are likely to become infected from those that are not. The method, as it is used, and as we have used it in these tests consists in injecting intracutaneously 0.1 cc. of a solution containing one-fiftieth of a minimum lethal dose of diphtheria toxin. As a control a similar amount of the same diluted toxin, heated previously for one hour at 60°C. is injected. The control is most essential because in a number of individuals a reaction is seen, generally within the first twenty-four hours, which is due undoubtedly to substances other than the toxin. This method is a quantitative test for neutralizing substances in the tissue fluids and blood of the person tested. If neutralizing substances are present the toxin is made inert. If they are not present local hyperaemia-oedema and even necrosis of a noticeable degree results from the presence of the free toxin. A concentration of toxin sufficient to cause marked necrosis is never used. We have made all our readings after forty-eight and seventy-two hours and called only those positive where the hyperaemia was most marked in the area injected with unheated toxin (8).

This particular time of year has not been one most suitable for obtaining cases of diphtheria for study. One of us recently has seen 3 cases and has obtained blood from them at the time of admission and before the treatment was given. The study of these cases has shown us that this method may be used for the

quantitative estimation of toxin just as the results of the experiments reported in this paper have shown that the method is suitable for a quantitative estimation of the neutralizing value of the blood of normal individuals.

It is evident, however, that this method finds application, with slight variation in the choice of the tissue, not only for the study of diphtheria but for the study of other toxins and antitoxic substances. The work with bacteria in relation to pyogenic infections has given many promising results but in spite of these results the great majority of these infections remain a menace. It has become evident that there is an urgent need for progress in new directions for the study of these diseases. While at Cornell University Medical College one of us noted a toxic substance capable of killing human tissues in the blood of a pneumonia patient. Toxic substances were also seen in individuals recovering from acute alcoholism. A more careful study of these cases may lead us to important conclusions. These latter facts have made it of greater interest to submit the report of these experiments for publication.

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ANTIBODY PRODUCTION IN RABBITS FOLLOWING INJECTION WITH PANCREATIC FERMENTS

HEINOSUKE WAGO

From the Laboratory of Preventive Medicine, University of Chicago

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Accumulated investigations give evidence that ferments when injected into suitable animals display antigenic properties in that they stimulate the production of specific antibodies. Thus:

Hildebrandt (1) in 1893, produced specific antibodies to the enzyme emulsin. Morgenroth (2) in 1899 and Briot (3) in 1900, were both successful in producing a specific antibody to rennin. In 1901 Achalme (4) produced certain specific antibodies to pancreatin, while Sachs (5) a year later produced similar antibodies to pepsin. In 1903 Levene and Stooky (6) increased the antitryptic action of serum by the experimental injection of pancrease extract, and Halpern (7) in 1911 claimed similar results from the injection of dog pancreas into dogs. Most recently (1914) Marras (8) obtained precipitating and complement-fixing antibodies to trypsin.

In the course of experiments directed toward further determination of the antibodies produced to pancreatic ferments, I have made observations that possess a bearing upon the production of antibodies in general and which it is the purpose of this paper to present.

The general line of experimentation was the injection of pancreatic ferments in various forms into rabbits, with a subsequent determination of the presence or absence of specific antibodies in the body fluid of the recipients. For this purpose I prepared pancreatin solutions as follows. Commercial pancreatin powder (Parke, Davis and Company) was added to physiological salt solution in the amount of 1 per cent by weight. After repeated shaking, the solution was filtered through hard-

pressed filters and finally through Berkefeld candles. The resulting filtrate was clear and sterile. Similar solutions were made of trypsin (Central Scientific Supply Company) and of amylopsin (Digestive Ferments Company). For injection, these solutions were employed intravenously, 5 cc. being introduced per kilo weight of animal.

The serum of animals receiving the injections of the above materials was tested from time to time for specific antibody content. The tests were directed especially toward the detection of precipitins, complement deviating antibodies, and of antibodies specifically inhibiting the ferment action of trypsin. The tests for precipitins were made against 1 cc. of the above 1 per cent ferment solutions: The complement deviation tests were made against twice the minimal activating dose of guinea pig serum for an immune serum specifically cytolytic for sheep erythrocytes: The antitrypsin tests were made against twice the amount of pancreatin or trypsin required to digest a carmine-stained fibrin flake, according to the method of Grützner (9).

Systematic injection of animals with the pancreatin showed that only after a multiplicity of injections was there a distinctly recognizable production of antibodies of any type. After six or more injections, however, a precipitin was demonstrable which reacted with solutions of pancreatin, of trypsin, and of amylopsin, and with all to about an equal degree. Reacting with such solutions, the immune sera also produced complement deviation.

Thus, rabbit 9 after receiving three 9 cc. injections of the 1 per cent pancreatin in six days, showed no distinct antibody content of its serum on the eighth day following the last injection. But after five additional similar injections, its serum finally displayed a marked content of precipitins for pancreatin solution; 0.00025 cc. effecting a distinct reaction. Complete complement deviation was produced by 0.025 cc. On the other hand, the serum displayed no specific power of inhibiting pancreatic digestion of fibrin. The antibodies formed therefore were clearly gained to admixed substances in the pancreatin preparation and not to the fibrinolytic ferment per se. The

results cited for rabbit 9 were approximated in all of eleven rabbits similarly injected.

The inability to produce antibodies in appreciable amount by a few injections of the relatively strong pancreatin solution, led to the examination of the urine as a suspected avenue of rapid excretion of the pancreatin, thus reducing its antigenic action. It was at once found that following intravenous injection, pancreatin is excreted in the urine in very large quantities. This applies not only to the ferment constituents themselves but to the admixed proteins as well. In general the urine of normal rabbits possesses no tryptic action, but within two hours after the intravenous injection of pancreatin as practiced in these experiments, the urine displayed a marked pancreatic action and continued to do so for forty-eight hours. Fifty-three rabbits were injected with the 1 per cent pancreatin solution and were killed after the duration of various time intervals the urine being at once drawn from the bladder with aseptic precautions. Forty-seven of the specimens thus obtained from animals killed in from two to forty-eight hours following the injection, displayed the power of completely digesting fibrin in vitro by the Grützner method, most of the urines producing the digestion when diluted tenfold. They also contained proteins of native pancreatin, as shown by their reaction with specific precipitins to these substances.

I next attempted to obviate this escape of the pancreatin antigens in the urine by injecting them in a less soluble form. To this end I prepared a pancreatin solution as follows:

> A 10 per cent solution of pancreatin was made in physiological salt solution, and after thorough shaking was filtered through hard-pressed paper. To the resulting filtrate was added 20 volumes of absolute alcohol and the mixture was allowed to stand thirty minutes with the resulting formation of a white precipitate. The mixture was then centrifugalized and the sediment was rapidly resuspended in an amount of salt solution making the concentration 2 per cent relative to the amount of pancreatin powder originally employed.

The pancreatin so treated with alcohol, retained its fibrinolytic value unmodified but when injected, it was excreted by the

urine much less extensively than unmodified pancreatin. The following experiment (table 1) shows that the alcohol treatment of the pancreatin did not diminish its fibrinolytic value.

Although as shown above, the fibrinolytic action of pancreatin was not modified by the alcohol treatment, its susceptibility to excretion by the kidneys was much reduced as shown in the following table (table 2).

The above table shows a greatly reduced excretion of the proteolytic ferment of pancreatin via the urine when the pancreatin is injected in an alcohol modified form. There was also

TABLE 1

Digestion of fibrin

1 PER CENT	A. UNMODIFIED PANCREATIN	B. ALCOHOL MODIFIED PANCREATIN
cc.		
1.0	Complete	Complete
0.75	Complete	Complete
0.5	Complete	Complete
0.35	Complete	Complete
0.25	Complete	Complete
0.15	Complete	Complete
0.1	Complete	Complete
0.075	Complete	Complete
0.05	Slight	Slight
0.035	Slight	Slight
0.025	0	0
0.015	0	0

a decreased excretion of the admixed pancreatin proteins as shown by the absence of a precipitin reaction with specific sera.

The antigenic value of the alcohol-modified pancreatin was next tested. To this end I employed alcohol-modified pancreatin for intravenous injection in experiments paralleled by the similar injection of unmodified pancreatin. Whereas in no instance was it possible to produce an appreciable amount of antibodies by a single injection of the unmodified pancreatin, the corresponding injection of the modified pancreatin invariably produced a very considerable amount of antibodies. Thus rabbit 14, which received a single injection of 10 cc. of the alcohol precipitated pancreatin, solution, displayed on the eighth day

such antibodies in its serum that 0.025 cc. of the serum produced a specific precipitin reaction and 0.035 cc. effected complete complement deviation. Rabbit 12, similarly injected but with unmodified pancreatin, showed no analogous antibody content of its serum. Simi ar differences were observed in all experiments where alcohol modified pancreatin injections were opposed to native pancreatin injections.

It is to be concluded, therefore, that a modification of the pancreatin rendering it less soluble and limiting its excretion by the urine, favors its power of stimulating the production of antibodies to the native proteins of pancreatin. Even with the al-

TABLE 2

Digestion of fibrin by twenty-four hour urine of rabbits

URINE	INJECTED WITH	
	A. Unmodified pancreatin	B. Alcohol modificd pancreatin
cc.		
0.1	Complete	Complete
0.075	Complete	Slight
0.05	Complete	0
0.035	Complete	0
0.025	Slight	0
0.015	0	0

cohol—modified pancreatin, however, no demonstrable antibody was formed specific in inhibiting the fibrinolytic ferment.

As a further means of favorably modifying the antibody production to the proteins of pancreatin, I employed injections of sodium iodoxybenzoate. Hektoen (10) and Arkin (11) showed that when this substance is injected intravenously immediately following the introduction of erythrocytes or bacteria as antigens, the antibody production is distinctly greater than when antigen alone is injected. Applying the general technique of the above workers, I injected 5 cc. of a $\frac{N}{20}$ sodium iodoxybenzoate solution into rabbits immediately following the introduction of native pancreatin as antigen. The results obtained were analogous to those recorded by Hektoen and by Arkin in that the animals receiving the iodoxybenzoate displayed a distinctly greater anti-

body production than the control animals not receiving the salt. The contrast was most clearly seen in animals which received but a single antigen injection. Such animals as received no iodoxybenzoate, produced no appreciable amount of antibodies, whereas all animals receiving the iodoxybenzoate produced a very distinct amount of precipitins. The following table (table 3) gives quantitative differences in the precipitin antibody content of the sera of two animals receiving the same injection of pancreatin, the one with and the other without subsequent injection of the sodium iodoxybenzoate. The tests were made on the eighth day following a single injection of 10 cc. of 1 per cent pancreatin.

TABLE 3

Effect of sodium iodoxybenzoate upon production of precipitins to pancreatin

IMMUNE SERUM	PRECIPITIN VALUE OF SERUM FROM	
	Rabbit A, receiving pancreatin only	Rabbit B, receiving pancreatin and iodoxybenzoate
cc.		
0.1	0	+++
0.075	0	+++
0.05	0	+++
0.035	0	+++
0.025	0	+++
0.015	0	++
0.01	0	0

From table 3 it is seen that whereas no specific precipitins were demonstrable in the rabbit receiving pancreatin alone, a very distinct content of precipitins was present in the serum of the rabbit receiving pancreatin and iodoxybenzoate.

The experiments thus far given show that an increased production of antibodies to pancreatin proteins may be induced in two ways: First, by an alcohol modification of pancreatin, rendering it less soluble; and secondly, by the introduction of sodium iodoxybenzoate into the recipient animal.

I next sought to determine whether or not the combination of these two favorable factors would yield an antibody production greater than that induced by either singly. The experi-

mental results showed that such was the case, namely, that the maximum antibody production was gained where the alcohol modified pancreatin was used as antigen and where the injection of the antigen in this form was accompanied by an injection of sodium iodoxybenzoate. The following table (table 4) presents a comparison of the amount of precipitins gained by the single

TABLE 4

Relative precipitin production to pancreatin

IMMUNE SERUM	PRECIPITIN REACTION BY SERUM FROM			
	A. Rabbit receiving unmodified pancreatin	B. Rabbit receiving alcohol-modified pancreatin	C. Rabbit receiving pancreatin with iodoxybenzoate	D. Rabbit receiving alcohol-modified pancreatin and iodoxybenzoate
cc.				
0.1	0	+	+	+
0.075	0	+	+	+
0.05	0	+	+	+
0.035	0	+	+	+
0.025	0	+	+	+
0.015	0	0	+	+
0.01	0	0	0	+
0.0075	0	0	0	+
0.005	0	0	0	+
0.0035	0	0	0	+
0.0025	0	0	0	+
0.0015	0	0	0	+
0.001		0	0	+
0.00075		0	0	+
0.0005		0	0	+
0.00035		0	0	+
0.00025		0	0	+
0.00015		0	0	+
0.0001		0	0	0

injection of unmodified pancreatin, of alcohol-modified pancreatin, of unmodified pancreatin plus iodoxybenzoate, and of alcohol-modified pancreatin plus iodoxybenzoate.

The above table (table 4) shows that whereas alcohol-modified pancreatin on the one hand, and unmodified pancreatin plus iodoxybenzoate on the other, have a greater antigenic value than unmodified pancreatin alone, a far greater antibody pro-

duction is effected by the combined use of alcohol-modified pancreatin and iodoxybenzoate. The figures tabulated refer to differences obtained in animals receiving a single injection of antigen. Similar relations hold however in animals receiving multiple injections and by employing the modified pancreatin and iodoxybenzoate in successive injections, sera were produced of which 0.000015 cc. effected the specific precipitin reaction. No sera of comparable value were obtained by any other procedure.

As the result therefore of the study here transmitted I have to conclude:

1. That the precipitins and complement deviating antibodies produced in response to pancreatin injection are distinct from such antibodies as may inhibit the proteolytic ferments of pancreatin. The former, as in the present experiments, may be produced in large amounts in the absence of the production of the latter in any degree whatsoever.

2. That following intravenous injection of pancreatin, the proteolytic ferments and proteins of pancreatin are extensively excreted by the urine.

3. That the antigenic value of pancreatin for stimulating the production of antibodies to the contained proteins is enhanced by an alcohol modification of the pancreatin.

4. That the production of precipitins to pancreatin as a soluble antigen is favorably influenced by the intravenous injection of sodium iodoxybenzoate.

5. That the production of precipitins to pancreatin may be enhanced to the greatest degree by employing alcohol-modified pancreatin as antigen and by accompanying its injection with that of sodium iodoxybenzoate.

I wish to express my thanks to Prof. Preston Kyes for many helpful suggestions.

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ON RED CELL GLOBULIN[1]

C. B. BENNETT AND CARL L. A. SCHMIDT

From the Department of Biochemistry and Pharmacology, University of California.

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A number of workers (1) have attempted to isolate from red cells the antigen which gives rise to a specific lysin and an agglutinin when red cells are repeatedly injected into an animal. These attempts have confined themselves chiefly to three constituents of the red cell: laked blood, the insoluble stroma and extracts by various lipoid solvents. On this subject Ford and Halsey (2) report that injection of either red cells, insoluble stroma or the water-soluble portion of red cells obtained by laking will give rise to a lysin and an agglutinin specific for the cell of the particular species used, these phenomena being inseparably connected. With the exception of hemoglobin, which is probably non-antigenic, no attempt has been made to separate from red cells a definite chemical entity which on injection will give rise to a lysin for the red cell.

It may be conceived that the antigen concerned in the production of hemolysis may be one of the following possibilities: (a) the protein complex which probably exists in red cells analogous to the complex which Hardy (3) assumes exists in blood serum and to which Robertson (4) has suggested the specificity of animal tissues and fluids may be due; (b) a single protein constituent of the red cell which may be separated in a pure state; (c) protein constituent occurring only in small quantities in the red cell in an analogous manner to the association of immune bodies with a particular fraction of blood serum and which cannot easily be separated; (d) lipoid constituent; (e) hemoglobin. The work

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of Bradley and Sansum (5) would indicate that hemoglobin is antigenic and specific for any particular species, but when it is recalled that no attempt was made adequately to purify the hemoglobin from the protein constituents of the red cells, these results cannot be accepted without serious question. Moreover, Ford and Halsey (2) were unable to produce immune bodies on repeated injections of purified hemoglobin. That the antigen is of a lipoidal nature as found by Bang and Forssman (6) and others has also been seriously questioned (7).

Our work, which concerns itself with the CO_2-precipitable globulin of red cells, was undertaken for a two-fold purpose: (1) to study the relation of this protein to the production of a lysin for red cells, (2) the relation of this globulin to the CO_2-globulin from the blood serum of the same species. The work was started by one of us (Bennett) some time ago and has since then been entirely repeated and extended.

This protein is mentioned by Kühne (8) and is probably related to the paraglobulin obtained from laked red cells by Woolridge (9) and Halliburton and Friend (10). For its preparation we proceeded as follows:

Oxalated blood from the ox was centrifuged, the serum was removed and the red cells were washed with normal saline nine times, the cells being thrown to the bottom by centrifuging and the supernatant fluid being withdrawn each time. The red cells were then laked with two volumes of water, and centrifuged to remove insoluble stroma and unhemolyzed corpuscles. This fluid was then brought to a dilution of ten parts of water to one of corpuscles and saturated with CO_2 until the globulin separated in large flakes. It was then repeatedly washed by decanting with distilled water until practically no further color of hemoglobin was apparent in the wash water. The suspended globulin was then centrifuged off, and the supernatant fluid removed, and to the compact mass sufficient solid NaCl was added to bring it to physiological concentration. On centrifuging off the gross particles, a semi solution of the globulin was obtained which however contained at this concentration some finely divided undissolved globulin. For the purposes of injection, phenol was

added to make a concentration of 0.5 per cent. Although the globulin is probably white we were unable completely to separate traces of hemoglobin, even after repeated washing. The globulin is insoluble in distilled water, but it is readily soluble in physiological salt solution. It gives a positive biuret, xanthoproteic and Millon's reaction. On boiling, coagulation occurs, and large flakes are precipitated. On standing for a long time in the presence of phenol, denaturization apparently slowly occurs. For the preparation of CO_2-globulin from ox serum we used the well-known method described by Quinan (11).

For the production of immune bodies two normal rabbits were each given 2 cc. doses of the concentrated globulin preparation intravenously for three successive days and after a five-day interval this was repeated. No symptoms were shown by the injected animals. Eleven days after the last injection the rabbits were bled and sera free from traces of hemoglobin were obtained. After the inactivation of the immune sera at 57°C. for one half hour to remove alexin, fixation experiments were carried out, both cell and serum globulin being used as antigen. The following amounts were used for the hemolytic system: antigen, 0.05 cc. of a 1:10 dilution (one-quarter of the minimal inhibiting dose); alexin 0.15 cc. of 10 per cent guinea pig sera (1.5 units); 0.2 cc. of 1:2000 rabbit vs. sheep red cells amboceptor; 0.2 cc. of a 5 per cent suspension of washed sheep cells; salt solution was added to bring the volume to 1 cc. When cell globulin was used as antigen, positive fixations were obtained for animal (1) in a dosage of 0.1 cc. of a 1:250 serum dilution and for animal (2) in a dosage of 0.2 cc. of a similar dilution. When serum globulin was used as antigen no fixations were obtained in any dilution of serum which in the absence of antigen was not inhibitory. The non-identity of the two globulins is thus apparent. This is in agreement with the work reported by Thomsen (12) who found that guinea pigs sensitized with serum alone did not react anaphylactically to the homologous red cells and vice versa.

To demonstrate the presence of red cell lysin in the sera of the immunized animals a system essentially similar to the above was used. A 5 per cent suspension of washed ox cells was used in a

dosage of 0.2 cc., 0.15 cc. of 10 per cent alexin, immune sera in varying dilutions, and salt solution to bring the volume to 1 cc. For the serum of animal (1) lysis occurred in a dosage of 0.1 cc. of a 1:1250 dilution and for animal (2) 0.3 cc. of 1:7250 serum dilution caused lysis of the red cells. On omitting alexin, agglutination of red cells occurred in a serum dilution of 1:250.

Since by using the CO_2-globulin of red cells as antigen we have obtained, on injection, antibodies similar to those obtained on injection of the entire constituents of the red cell, we cannot assume that the protein complex of the red cell is necessary for the production of immune bodies, and excluding the lipoids and hemoglobin as factors we are left with two possibilities viz., the CO_2-globulin or another substance which cannot easily be separated from it. A choice of these is not yet possible. Of interest in this connection is the report of Quinan (13) that the substance occurring in serum which causes lysis of red cells is neither the soluble nor insoluble globulin nor serum albumin. He regards the substance as being probably a ferment.

SUMMARY

1. It was found that the sera of animals immunized with CO_2-globulin from washed red cells contained immune bodies specific for this globulin and not for the CO_2-globulin from the homologous serum and substances which in the presence of alexin will cause lysis of the homologous red cells and in the absence of alexin, agglutination of these cells.

2. It cannot be definitely stated whether the CO_2-globulin from red cells is the antigen concerned in the production of a lysin for the homologous red cell or a substance intimately associated with it. The possibilities are discussed.

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STUDIES IN OSMOTIC PRESSURE

I. THE MECHANISM OF BORIC ACID HEMOLYSIS

MITSUJI KOSAKAI

From the Department of Bacteriology of Cornell University Medical College, New York City

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Of the long list of the studies of the phenomena of the hemolysis almost all have been concerned with the investigation of the properties of the various hemolytic agents, very few having pursued the question of the actual force that brings about the destruction of the corpuscles. Indeed, this ultimate force is known in but one of the many forms of the process of hemolysis that resulting from hypotonicity of the medium of suspension; that is, osmotic pressure.

In the course of some experiments upon the preservation of blood corpuscles with boric acid A. F. Coca made the following observations:[1] boric acid in dry form or in solution may be mixed with blood in a proportion of $1\frac{1}{2}$ per cent, or less, of the dry substance without causing hemolysis. If blood that has been in contact with boric acid for a time is suddenly mixed with one or more volumes of 0.9 per cent NaCL solution, immediate complete hemolysis occurs. If the blood that has been treated with boric acid is mixed with more concentrated solutions of sodium chloride no hemolysis takes place.

As the easily controlled conditions leading to this phenomenon appeared to be favorable for the discovery of the ultimate mechanism of the hemolytic process, the present further study of it was undertaken.

[1] Unpublished.

TECHNIC

The corpuscle suspension used throughout this investigation was prepared from oxalated sheep's blood by washing the latter three times in physiological (0.9 per cent) NaCL solution and diluting the corpuscular sediment with the same solution up to the original volume of the blood taken.

The concentration of the solutions of all of the substances employed was determined by titration so far as methods of titration for this purpose were available.

The glass tubes used for the hemolysis tests were of ordinary American glass and they measured 7 cm. in length by about 1 cm. in diameter.

The experiments were carried out at room temperature (20° to 24°C). The influence of ordinary changes of temperature upon the results was quite negligible.

The direct effect of boric acid upon the corpuscles was first examined and it was found that, in the stronger concentrations, this substance is capable, after a period of time, the length of which is influenced directly by its concentration, of producing complete or nearly complete laking. The protocol of an experiment showing the direct hemolytic action of boric acid is presented in table 1.

That this direct hemolytic effect is not due to the acid quality of the substance; that is, to the action of hydrogen ions, seems deducible from the fact that boric acid is not dissociated in solution and also from the absence of methemoglobin formation, which is always observed after hemolysis with mineral acids and dissociable organic acids.

The preceding experiment does not exclude the possibility that, although the direct boric acid hemolysis depends on the *concentration* of the reagent in the original mixture, the phenomenon is influenced, also, by the quantitative relation between the corpuscles and the boric acid. This question was investigated by preparing two mixtures of equal volume containing an identical amount of boric acid but different amounts of corpuscles. The boric acid concentration used was such that

the usual amount of the corpuscle suspension (0.05 cc., tube A) was not completely hemolysed.

If the degree of hemolysis were affected by the quantitative relation between the corpuscles and the reagent, then less hemolysis would be expected in the mixtures containing the larger amounts of corpuscles (that is 0.1 cc., tube B). However, the degree of hemolysis was identical in the two mixtures. On the other hand, the hemolytic effect was distinctly less in a mixture in which the quantitative relationship between cor-

TABLE 1

Showing the direct hemolytic action of boric acid

	NUMBER OF TEST TUBE				
	I	II	III	IV	V
Boric acid solution (3.5 per cent), cc.	0.3	0.5	0.95	1.95	2.95
Physiological salt solution (cc.)	0.65	0.45			
Blood suspension (cc.)	0.05	0.05	0.05	0.05	0.05
	Degree of resulting hemolysis				
8 minutes	0	0	0	0	c
12 minutes	0	0	0	c	c
2.5 hours	0	tr	alc	c	c
20 hours	0	tr	alc	c	c

In all of the tables the following abbreviations are used to indicate the degree of the resulting hemolysis: c = complete, alc = almost complete, v.st. = very strong, st = strong, w = weak, v.w = very weak, tr = trace, 0 = no hemolysis.

puscles and boric acid was the same as that of mixture B but in which the concentration of the reagent was only half as great as in the latter mixture.

From the results of the first experiment it is seen, furthermore, that in the lesser concentrations (tubes 1 and 2) boric acid exerts little or no hemolytic action. These concentrations were, therefore, suitable for the demonstration of the phenomenon of hemolysis observed by Coca. For this purpose a constant amount of corpuscular suspension was mixed with boric acid in the strongest concentration that was not directly hemo-

lytic and also in three lesser concentrations. After five minutes an equal volume (1 cc.) of 0.9 per cent NaCI solution was added to each mixture and the tubes were at once vigorously shaken.

The results of this experiment are presented in table 2.

It is seen that complete hemolysis occurred only in the mixture containing the stronger concentration of the boric acid; that is, *where the relative change in the concentration of that substance caused by the addition of the salt solution was greatest.* Furthermore, a significant characteristic of the boric acid hemolysis[2] is seen in the results of this experiment, in the fact that *the full hemolytic action elicited by the procedure is exerted*

TABLE 2

	NUMBER OF TEST TUBE			
	I	II	III	IV
Boric acid solution (3.5 per cent) (cc.)	0.5	0.4	0.3	0.2
Physiological salt solution (cc.)	0.45	0.55	0.65	0.75
Blood suspension (cc.)	0.05	0.05	0.05	0.05
After five minutes, 1 cc. of physiological salt solution is added into each tube				
Result: At once	c	w	tr	0
Result: After ten hours	c	w	tr	0

within a few seconds, not the least further hemolysis occurring in any of the tubes during the succeeding ten hours.

Since the cause of the hemolysis appeared to depend upon the sudden change of the concentration of the boric acid in the medium of suspension of the corpuscles it seemed possible that the addition of more than an equal volume of salt solution would cause hemolysis, also, in the mixtures containing lesser concentrations of boric acid and this was found to be true. When to the mixtures in tubes 2 and 3 in table 2 several volumes of 0.9 per cent NaCI solution were added complete hemolysis took place in both instances.

[2] In the further discussions in this paper the term "boric acid hemolysis" refers to the phenomenon of hemolysis observed by Coca, not to the direct hemolysis produced by the concentrated reagent.

In view of these latter results it could be anticipated that the addition to the mixture in tube 1 of less than an equal volume of salt solution could result in a correspondingly lesser degree of hemolysis. That this is true is shown in the protocol presented in table 3.

It is seen that when a half volume of salt solution was added to the mixture only weak hemolysis occurred, the addition of a three-tenths volume causing only a trace of hemolysis.

Similarly, it was anticipated that if the mixtures in the tubes 2 and 3 of table 2 were centrifuged and the supernatant fluid decanted, hemolysis could then be produced by shaking the sediment in a relatively small volume of NaCl solution—one

TABLE 3

	NUMBER OF TEST TUBE		
	I	II	III
Boric acid solution (3.5 per cent) (cc.)	0.5	0.5	0.5
Physiological salt solution (cc.)	0.45	0.45	0.45
Blood suspension (cc.)	0.05	0.05	0.05
After five minutes was added to	I, 1.0 cc. of physiological salt solution; to II, 0.5 cc. of physiological salt solution; to III, 0.3 cc. of physiological salt solution.		
Result: At once	c	w	tr
Result: After ten hours	c	w	tr

which, without centrifugation, would fail to cause laking. The results of such an experiment, which are given in table 4, show this to be the case.

That the hemolysis produced by the addition of salt solution to blood that has been treated with boric acid is not due to a specific action of sodium chloride is demonstrated by the fact that hemolysis occurs as in all of these circumstances if, instead of the solution of sodium chloride isotonic solutions of other salts or of sugar are added to the blood corpuscle and boric acid mixtures. Indeed, the same phenomenon is observed upon the addition of sheep's serum to those mixtures.

The data in hand made it appear possible that the *concentration* of the boric acid rather than its absolute quantity in

the mixtures determined its hemolytic effect. That this is true, was demonstrated by repeating the experiment referred to in table 4 but using a quantity of salt solution in preparing the original mixtures such that the total volume was always 5 cc. instead of 1 cc. After these mixtures had stood over night none of the corpuscular sediments obtained by centrifugation and decantation was hemolysed when rapidly mixed with even a large volume of 0.9 per cent NaCl solution.

TABLE 4

TUBE I	TUBE II	TUBE III
Boric acid (3.5 per cent) ... 0.3 cc. Physiological salt solution ... 0.65 Blood suspension ... 0.05	Same as I	Same as I
After five minutes, added 2 cc. of physiological salt solution, shaken rapidly Result = complete hemolysis	After five minutes, ten times 0.2 cc. of physiological salt solution added with intervals of thirty seconds, each time shaken Result = no hemolysis	
	Then this test tube centrifuged, into sediment added 0.5 cc. of physiological salt solution, shaken rapidly Result = no hemolysis	After five minutes centrifuged, into sediment added 0.5 cc. of physiological salt solution, shaken rapidly Result = complete hemolysis

The experiments thus far had elicited the following facts:

1. The boric acid hemolysis is exerted to its full extent, in whatever degree, within a few seconds, no further hemolysis thereafter taking place.

2. The boric acid hemolysis depends on the concentration, not on the absolute amount of the reagent with reference to a constant quantity of corpuscles.

3. The boric acid hemolysis occurs after a sudden lowering of the concentration of the reagent in the medium of suspension of the corpuscles.

The only force that is known to be developed under the foregoing conditions is that of osmotic pressure, which, of course, must be assumed to act upon a limiting corpuscular cell membrane and the further evidence, which will be presented, leaves no reasonable doubt that this force is, in fact, the ultimate cause of the phenomenon of hemolysis that we are studying.

The assumption that the boric acid hemolysis is an effect of osmotic pressure necessitates the further assumption that the reagent is able to permeate the corpuscular membrane. As is well known (1) the corpuscular membrane is permeable to many substances and impermeable to others. Since boric acid had not yet been studied in this respect, it was necessary to determine the question by experiment. For this purpose the method of Hedin was employed as follows: To 10 cc. of washed sheep's blood, made up to the original volume with physiological saline solution, were added 10 cc. of physiological saline solution containing 3.5 per cent of boric acid. After 10 minutes this mixture was centrifuged and the supernatant fluid was compared with a mixture of 10 cc. of physiological saline solution and 10 cc. of the boric acid-saline solution as to its osmotic concentration.

If the boric acid was capable of entering the corpuscles and was present there in the same concentration as in the medium of suspension, then the osmotic concentration in the fluid medium was, of necessity, the same as that of the mixture without the corpuscles. On the other hand, if the corpuscular membrane was impermeable or incompletely permeable to the boric acid, then the osmotic concentration in the medium of suspension must have been greater than it was in the mixture without the corpuscles. The osmotic concentration in the two fluids was determined with the cryoscopic method, with the following result:

1. Supernatant fluid of corpuscle mixture.....1.120
2. Control mixture without corpuscles.....1.122

The distribution of the boric acid in the suspension of corpuscles is, thus, uniform throughout the corpuscles and the medium of suspension.

GRADUAL ADDITION OF PHYSIOLOGICAL SALINE SOLUTION TO "BORATED" CORPUSCLES

If the force that is operative in the boric acid hemolysis is purely that of osmotic pressure and if the corpuscles are not directly injured by the mere contact with the reagent, it should be possible, by the gradual addition of a volume of salt solution that, otherwise, is hemolytic, to bring about a slow diffusion of the intracorpuscular boric acid into the surrounding medium without the production of hemolysis. The difference between the intracorpuscular and the extracorpuscular concentration of the boric acid should, by this procedure, easily be kept below the point at which the assumed destructive osmotic pressure is developed. The protocol of the experiment that was conducted according to this plan is presented in table 4.

The result of the experiment is in harmony with the "osmotic pressure" theory of the boric acid hemolysis and it demonstrates that the hemolytic effect is not caused by a direct injury of the corpuscles by the reagent.

THE INHIBITION OF THE BORIC ACID HEMOLYSIS WITH CONCENTRATED SOLUTIONS OF ELECTROLYTES AND NON-ELECTROLYTES

The already cited observation of Coca, that the boric acid hemolysis can be inhibited if, in the second step of the procedure concentrated NaCl solution is substituted for the 0.9 per cent NaCI solution, is also compatible with the osmotic pressure theory. This phenomenon was subjected to a quantitative study; the protocol of the preliminary experiments is presented in table 5.

It is seen that corpuscles which have been treated with boric acid in the manner indicated can be mixed suddenly with 1 cc. of a 1.4 per cent or more concentrated solutions of sodium chlorid

without undergoing the least degree of hemolysis. In concentrations of 1 per cent or less the corpuscles are completely laked.

In terms of the osmotic pressure theory it could be assumed that certain concentrations of sodium chlorid are able to offer a counter osmotic pressure upon the external aspect of the corpuscular membrane which neutralizes that developed by the boric acid within the cell. It could be assumed, furthermore, that those concentrations of sodium chlorid need not prevent the diffusion of the boric acid out of the corpuscles and experimental examination of this question, the protocol of which is presented in table 6, shows that such is the case.

TABLE 5

Each tube contains: Boric acid solution (3.5 per cent), 0.3 cc.; physiological salt solution, 0.65 cc.; blood suspension 0.05 cc. After five minutes, all centrifuged, and 1.0 cc. of the solutions of sodium chloride added.

	NUMBER OF TEST TUBE							
	I	II	III	IV	V	VI	VII	VIII
Concentration of sodium chloride (per cent)	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6
Result: At once	c	c	v.st	w	tr	0	0	0
Result: After two hours	c	c	v.st	w	tr	0	0	0

The insensitiveness of the corpuscles, after the second centrifugation, to sudden immersion in physiological saline solution indicates that the concentration of boric acid remaining in them was no longer great enough to develop a destructive osmotic pressure. Moreover, the result of this experiment furnishes clear evidence that the boric acid hemolysis is not due to any organic change in the corpuscles caused by a chemical action of the reagent.

In order to avoid confusion resulting from discrepancies that may appear to exist, with respect to hemolytic effect, in the quantitative relations in the different protocols, it may be stated that the different specimens of sheep's corpuscles have been found to be differently susceptible to the hemolytic influence of boric acid that we are considering; that is, some speci-

mens of blood could be completely hemolysed after treatment with concentrations of boric acid, which, with other specimens of blood, induced only a partial hemolysis. This factor has sometimes interfered with a direct comparison of the results in the different protocols, but it has, in no way, detracted from the conclusions of the study, which have been based on individual experiments.

If the absence of hemolysis upon the immersion of borated corpuscles in a concentrated solution of sodium chlorid is due

TABLE 6

Each tube contains: Boric acid solution (3.5 per cent), 0.3 cc.; physiological salt solution, 0.65 cc.; blood suspension, 0.05 cc. After five minutes all tubes were centrifuged and the supernatant fluid in each was decanted

	NUMBER OF TEST TUBE			
	I	II	III	IV
Solution added	Physiological salt solution 1.0 cc.	1.5 per cent NaCl 1.0 cc.	2.0 per cent NaCl 1.0 cc.	2.5 per cent NaCl 1.0 cc.
Result	c	0	0	0
After five minutes tubes II, III and IV again centrifuged and the sediment in each was mixed suddenly				
Physiological salt solution		3 cc.	3 cc.	3 cc.
Result		0	0	0

to a counter osmotic pressure developed against the outer surface of the corpuscular membrane, then a similar effect must be producible with suitably concentrated solutions of other substances capable of inducing osmotic pressure. Furthermore, if the theory under consideration is correct, it must be possible to demonstrate that the minimal non-hemolytic concentrations of all such substances for corpuscles that have been treated with a certain concentration of boric acid actually exert the same osmotic pressure. In the succeeding experiments these requirements are fully satisfied and the concor-

dant results permit the definite conclusion that *the force operative in the boric acid hemolysis is, in fact, that of osmotic pressure.*

In these experiments the treatment of the corpuscles was always carried out in a volume of 1 cc. of the different concentrations of the boric acid. The concentration of the boric acid is usually indicated in the tables by the amount of a 3.5 per cent solution of that reagent that was contained in the treating mixture. For example, where the amount of boric acid is given as 0.15, this means that in making that mixture 0.15 cc. of 3.5 per cent of boric acid dissolved in physiological saline solution were mixed with 0.8 cc. of physiological saline solution and to this mixture were added 0.05 cc. of blood suspension.

In order to determine the minimal non-hemolytic concentration of the different substances, five or six identical mixtures of blood and boric acid were prepared and after five minutes the mixtures were centrifuged and the supernatant fluid was completely removed with a capillary pipet. With the sediment of each tube was rapidly mixed (by shaking) one cubic centimeter of the different concentrations of the substance under examination. Within a few minutes these final mixtures were centrifiged and the degree of the resulting hemolysis was noted according to the degree in which the supernatant fluid was tinged with hemoglobin. The minimal non-hemolytic concentration was taken as the lowest with which no tinging of the supernatant fluid resulted. The relative osmotic pressure of the various concentrations was determined with the usual cryoscopic method, the results of these examinations being recorded in the tables under the customary designation.

In a preliminary experiment the relation of the osmotic pressure of the treating mixture to that of the minimal inhibiting concentration of pure sodium chlorid and of boric acid dissolved in physiological sodium chlorid was studied and, as the tabulated protocols (tables XIII and XIIIa) show, it was found that the "minimal inhibiting pressure" was slightly, but consistently lower than the "treating pressure." It is impossible to interpret this difference without exact information as to the balance of osmotic pressure between the normal corpuscular

contents and the "isotonic" saline solution in which the boric acid, used in the experiment, had been dissolved. A part if not all of the difference represents variation due to experimental error.

It is seen, furthermore, that for corpuscles that have been similarly treated with boric acid the minimal non-hemolytic

TABLE 7

Showing the method of determining the minimal concentrations of a substance (NaCl) *which are capable of preventing the boric acid hemolysis*

	NUMBER OF GROUP							
	I	II	III	IV	V	VI	VII	VIII
Amount of boric acid solution (3.5 per cent) used for treatment.....	0.15	0.2	0.25	0.3	0.35	0.4	0.45	0.5
Solution of NaCl, per cent	Degree of resulting hemolysis							
0.9	st	c						
1.0	w	st	alc					
1.1	0	w	st	alc				
1.2	0	0	w	st	c			
1.3	0	0	0	w	st	c		
1.4		0	0	0	w	st	c	
1.5				0	0	w	alc	
1.6					0	tr	st	alc
1.7						0	v.w	st
1.8						0	0	v.w
1.9							0	0
2.0								0

concentration of sodium chlorid and of boric acid are found, with the method employed, to be of practically identical "osmotic concentration."

In two further series of tests, the results of which are presented in tables 9 and 10, the "osmotic concentration" of the minimal non-hemolytic concentrations of four other substances was determined for differently treated corpuscles. As different

TABLE 8

Relation of the concentration of boric acid used in treatment to the minimal inhibiting concentrations of sodium chloride and boric acid

TREATING CONCENTRATION OF BORIC ACID			INHIBITING CONCENTRATION			
Amount of a 3.5 per cent solution in 1.0 cc. of original mixture	Per cent	Δ	NaCl		Boric acid in physiological salt solution	
			Per cent	Δ	Per cent	Δ
cc.						
0.5	1.75	1.115	1.85	1.095	1.54	1.07
0.45	1.575	1.075	1.75	1.05	1.40	1.00
0.4	1.4	1.00	1.62	0.97	1.275	0.95
0.35	1.225	0.94	1.5	0.91	1.05	0.88
0.3	1.05	0.88	1.4	0.85	0.945	0.84
0.25	0.875	0.835	1.3	0.83	0.77	0.80
0.2	0.7	0.78	1.18	0.74	0.595	0.75
0.15	0.525	0.73	1.08	0.69	0.42	0.67

TABLE 9

Relative osmotic concentration of the minimal non-hemolytic concentrations of sodium chlorid, barium chlorid and cane sugar

AMOUNT OF 3.5 PER CENT BORIC ACID USED FOR TREATING THE CORPUSCLES	NON-HEMOLYTIC CONCENTRATION OF					
	NaCl		$BaCl_2$		Cane sugar	
	Per cent	Δ	Per cent	Δ	Per cent	Δ
cc.						
0.2	1.2	0.749	3.7	0.724	11.0	0.722
0.3	1.4	0.850	4.4	0.858	13.0	0.866
0.4	1.6	0.950	4.9	0.971	14.0	0.984
0.5	1.8	1.082	5.4	1.076	15.0	1.125

TABLE 10

Relative osmotic concentration of the minimal non-hemolytic concentrations of ammonium chlorid and glycerin

AMOUNT OF 3.5 PER CENT BORIC ACID USED FOR TREATING THE CORPUSCLES	NON-HEMOLYTIC CONCENTRATION OF			
	NH_4Cl		Glycerin	
	Per cent	Δ	Per cent	Δ
cc.				
0.2	1.2	0.801	2.9	0.794
0.4	1.5	1.010	3.8	1.041

specimens of corpuscles were used in the two series of tests, the results in the two tests are not concordant. Those of table 9 agree with those of table 8, both of these disagreeing with those of table 10. However, the results included within each table are concordant among themselves and they allow no reasonable doubt that *the prevention of the boric acid hemolysis with concentrated solution is due to a counter osmotic mechanism acting upon the outer surface of the corpuscles.*

SUMMARY

After blood corpuscles have been treated for a short time with certain concentrations of boric acid that are not directly injurious to those cells, the sudden immersion of the treated corpuscles in a physiological solution of sodium chloride causes their complete hemolysis.

This "boric acid hemolysis" does not occur if the addition of the physiological saline solution is made gradually or if the corpuscles are immersed, even suddenly, in more concentrated solutions of sodium chloride or of other non-hemolytic substances.

That the destructive force responsible for this form of hemolysis is that of "osmotic pressure" is shown by the fact that the minimal non-hemolytic concentrations of all of the substances examined were found to be of identical "osmotic concentration."

REFERENCE

(1) Hedin: Grundzüge der physikalischen Chemie, Wiesbaden, 1915, p. 22.

STUDIES IN OSMOTIC PRESSURE

II. THE NATURE OF OSMOTIC PRESSURE

MITSUJI KOSAKAI

From the Department of Bacteriology of Cornell University Medical College, New York City

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The laws governing the development of osmotic pressure and the effects produced by that agency upon animal and vegetable cells have been exactly determined by experimental study. The nature of the force of osmotic pressure, on the other hand, has, of necessity, been only surmised, because of the limitations hitherto surrounding the experimental study of that force, and the ideas concerning this question are based either on theoretical grounds or on the observations upon the effects of osmotic pressure.

In the writings on the subject of osmotic pressure two conceptions of the nature of that force are found.

The first conception is drawn from the well known demonstration by van't Hoff of the agreement between the laws governing gas pressure and those governing the development of osmotic pressure. According to this conception, osmotic pressure, corresponding with the pressure of the gases, is exerted by the molecules of the dissolved substance (solute), these being thought of as continually "bombarding" the surface of the limiting membrane.

Thus, Hedin (1) writes "da ferner der Zucker durch die Membran nicht passieren kann, so übt der Zucker gegen die Membran einen gewissen Druck aus. Dieser wird der *Osmotische Druck* der eingeschlossenen Lösung genannt."

Wells (2) says "since osmotic pressure, exactly like gas pressure, is presumably produced by the bombarding of the walls of the container by particles in solution. . . . ."

Lewis (3) writes "It seems reasonable to suppose, therefore, that when diffusion of a solute does occur in a given direction it is due to the osmotic pressure acting as the driving force. Of course we cannot speak of the osmotic pressure of the solvent, but simply of the solute, since the concentration of the solute corresponds to gas concentration."

Nernst (4) writes "it must happen, of course, that the sugar will exert a *pressure* on the partition, which opposes its endeavor to fill the whole solution." The further exposition by this author leaves no doubt that he looks upon osmotic pressure as being directly exerted by the solute.

The second conception of the nature of the force of osmotic pressure is expressed by van't Hoff (5) as follows:

> In order clearly to realize the quantity referred to as osmotic pressure, imagine a vessel completely full of an aqueous solution of sugar, placed in water. If it be conceived that the solid walls of this vessel are permeable to water but impermeable to the dissolved sugar, then, owing to the attraction of the solution for water, water will enter the vessel up to a certain limit, thereby increasing the pressure on the walls of the vessel [inside]. Equilibrium then ensues owing to the pressure resisting further entry of the water. This pressure we have termed osmotic pressure.

This conception ignores the dissolved substances as directly exerting the force that we are studying and looks upon the latter as merely the pressure developed by the accumulation of the water which diffuses through the semipermeable membrane into the solution containing the greater concentration of molecules and dissociated ions. The view just stated is not without adherents among the investigators of the subject of osmosis but it has received almost no consideration in the published treatises.

The preceding study of the boric acid hemolysis (6) had revealed conditions under which the development and effect of osmotic pressure can be exactly controlled and observed under varying quantitative relationships.

In previous investigations Eisenberg (7) had observed the laking of the corpuscles with formalin and urea under circumstances, which, so far as they were studied by him, coincide with those which we have found to control the phenomenon of boric acid hemolysis and we had observed a similar hemolytic action on the part of glycerin and ammonium chloride.

As will be presently set forth, a comparative study of the quantitative and time-relationships that govern the hemolytic effect of some of these reagents, has revealed facts which can be explained only with the assumption that *osmotic pressure is not a direct property of a solute but is developed indirectly, as a result of a process of diffusion, by the accumulation of water on one side of a semipermeable membrane.*

Eisenberg observed that corpuscles which had been in contact with formaldehyde in a concentration below that capable of "fixing" them were immediately laked on being suddenly immersed in isotonic saline solution. This hemolytic effect did not occur if the treated corpuscles were immersed in more concentrated salt solution. Eisenberg concluded that the formalin hemolysis is not a direct effect of the reagent. He considered the phenomenon as a "water hemolysis" but he adduced no experimental support for such assumption.

Eisenberg's observation of the urea hemolysis (in the lesser concentration of that substance in physiological saline solution) was confined to the mere fact that that effect is produced by a sudden immersion of the treated corpuscles in isotonic salt solution. Eisenberg excluded osmotic pressure as a cause of the urea hemolysis, because of the fact that with the lesser concentration of the reagent a longer contact was followed by a stronger hemolytic effect than a shorter contact. This fact, Eisenberg thought, pointed to a direct action of the urea.

The first experiments of the present study were designed for the purpose of determining whether the hemolytic action of formaldehyde and of urea were, like that of boric acid, the result of osmotic pressure and five criteria were used together in arriving at a conclusion as to that question. The first of these had already been applied by Eisenberg in his observation

that the hemolytic action of both substances was developed merely by a reduction in the concentration of the substance in the medium of suspension; the second criterion was the absence of hemolysis when the treated corpuscles were suddenly immersed in concentrated salt solutions; the third criterion was the disappearance of the peculiar sensitiveness of the treated corpuscles to immersion in physiological salt solution after being washed in concentrated salt solution; the fourth criterion was the absence of hemolysis when to the treated corpuscles a hemolytic volume of physiological salt solution was added not all at once, but gradually; and the fifth criterion was the demonstration of the permeability of the corpuscular membrane to both substances with the cryoscopic method of Hedin.

In all of these five respects the corpuscles treated with either formaldehyde or urea[1] behaved exactly like those treated with boric acid. Hence the conclusion is warranted that the hemolysis produced by the former two substances is, like that of boric acid, a result of osmotic pressure.

In order to make a comparative study of the osmotic hemolysis produced by the three selected reagents the concentrations were determined in which they all produce the same degree of hemolysis upon an arbitrarily selected, uniform diminution of those concentrations. It was found that if 0.05 cc. of corpuscular suspension were treated for ten minutes with 0.4 cc of 3.5 per cent boric acid or 0.4 cc. of 4 per cent formaldehyde or 0.8 cc. of 10 per cent urea, the total volume in each case being 1 cc., the sudden addition of 1 cc. of physiological salt solution would produce very strong hemolysis, while the addition of 2 cc. of that solution would cause complete hemolysis in all of the mixtures.

Under the condition of these comparative tests the assumption is justified that in all three instances, where the same degree of hemolysis was produced, the corpuscles were being subjected to the same degree of osmotic pressure.

[1]For a generous supply of urea of highest purity the author is indebted to Dr. William J. Gies of the College of Physicians and Surgeons in New York City.

On the basis of these tests, the same relative concentrations were used in the subsequent comparative study.

It was found that the osmotic concentrations of the mixtures containing 0.2 cc. of 3.5 per cent boric acid, or 0.2 cc. of 4 per cent formaldehyde or 0.4 cc. of 10 per cent urea were respectively $\Delta = 0.780$, 1.091 and 1.91; and that the corresponding changes in concentration in the comparative hemolytic experiment were as follows:

	CONCENTRATION OF THE SUBSTANCE IN THE TREATING MIXTURE	CONCENTRATION OF THE SUBSTANCE IN THE MIXTURE AFTER ADDITION OF THE MINIMAL HEMOLYTIC AMOUNT OF PHYSIOLOGICAL SALT SOLUTION
	per cent	*per cent*
Boric acid, 3.5 per cent, 0.2 cc	0.700	0.378
Formaldehyde, 4 per cent, 0.2 cc	0.800	0.267
Urea, 10 per cent, 0.4 cc	4.000	1.3

It is evident that if the destructive osmotic effect that we are studying is exerted directly by the molecules of the different substances, it should be expected that the solutions of the three substances which produce the same hemolytic effect would be found, by the cryoscopic method, to be of the same osmotic concentration; furthermore, it should be expected that a constant relation would be found between the concentration of each substance with which the corpuscles were treated and that of the respective mixture, after the minimal hemolytic quantity of physiological saline solution had been added.

However, in the experiments that were undertaken to determine this question, neither of these two requirements was satisfied.

It is seen that although the osmotic concentration of the mixture of boric acid used in the treatment of the corpuscles is considerably less than that of the other two substances the same hemolytic effect was produced by a change in the concentration of the reagent which was *much less* in the case of boric acid than it was in the other two substances; that is, *exactly the reverse* of what would have been expected if the osmotic force is exerted directly by the molecules of the reagent.

A comparison of the results obtained with formaldehyde and with urea shows a close correspondence in the ratios between the concentration used for treating the corpuscles and that resulting upon the addition of the minimal hemolytic amount of physiological saline solution. In view of the fact that the osmotic concentration of the mixture of formaldehyde used in treating the corpuscles was only about half as great as that of the treating mixture of urea a correspondingly *greater lowering of the concentration of* the former reagent should have

TABLE 1

Determination of the ratio of the final concentration to the original concentration of the reagent in boric acid hemolysis

	NUMBER OF TEST TUBE			
	I	II	III	IV
Boric acid solution (3.5 per cent) (cc.)	0.1	0.1	0.1	0.1
Physiological salt solution (cc.)	0.25	0.35	0.45	0.55
Blood suspension (cc.)	0.05	0.05	0.05	0.05
Whole volume (cc.)	0.4	0.5	0.6	0.7
Percentage concentration of boric acid in the treating mixtures	0.875	0.7	0.583	0.5
Minimal hemolytic amount of physiological salt solution, added after five minutes contact without previous centrifugation (cc.)	0.4	0.65	0.9	1.4
Final percentage concentration of boric acid	0.438	0.304	0.233	0.167

been thought necessary to the production of an identical hemolytic effect in the two mixtures.

This latter contention is verified by an examination of the protocols of three series of tests that were carried out with diminishing concentrations of boric acid, formaldehyde and urea respectively. These protocols are presented in tables 1, 2 and 3.

In all of these three experiments it is evident that, as the concentration of the reagent that was used for treating the corpuscles diminishes, the ratio between that concentration and the final concentration rapidly increases.

TABLE 2

Determination of the ratio of the final concentration to the original concentration of the reagent in formaldehyde hemolysis

	NUMBER OF TEST TUBE			
	I	II	III	IV
Formaldehyde solution (4 per cent) (cc.)	0.1	0.1	0.1	0.1
Physiological salt solution (cc.)	0.25	0.35	0.45	0.55
Blood suspension (cc.)	0.05	0.05	0.05	0.05
Whole volume (cc.)	0.4	0.5	0.6	0.7
Percentage concentration of formaldehyde in the treating mixtures	1.0	0.8	0.666	0.572
Minimal hemolytic amount of physiological salt solution, added after five minutes contact without previous centrifugation (cc.)	1.0	1.6	2.2	4.5
Final percentage concentration of formaldehyde	0.286	0.19	0.143	0.077

TABLE 3

Determination of the ratio of the final concentration to the original concentration of the reagent in urea hemolysis

	NUMBER OF TEST TUBE			
	I	II	III	IV
Urea solution (10 per cent) (cc.)	0.2	0.2	0.2	0.2
Physiological salt solution (cc.)	0.15	0.25	0.35	0.45
Blood suspension (cc.)	0.05	0.05	0.05	0.05
Whole volume (cc.)	0.4	0.5	0.6	0.7
Percentage concentration of urea in the treating mixtures	5.0	4.0	3.33	2.86
Minimal hemolytic amount of physiological salt solution, added after five minutes contact without previous centrifugation (cc.)	0.85	1.3	2.8	9.0
Final percentage concentration of urea	1.6	1.11	0.59	0.206

The experimental facts thus far adduced in the comparative study of the osmotic hemolysis produced by the three selected reagents are incompatible with the theory that osmotic pressure is exerted directly by the molecules of the solute.

They demonstrate that in circumstances under which the three substances are allowed to act upon the corpuscular membrane in identical "osmotic concentration" the quantitative effect is by no means identical in the three instances. Under such circumstances the effect produced by boric acid is greater than that of formaldehyde and that of the latter substance is in turn, greater than that of urea.

These differences are made further apparent by an analysis of the results presented in tables 1, 2 and 3.

As the treating concentration of the three substances is diminished the ratio between that concentration and the final concentration (that is, the concentration of the substance in the mixture after the addition of the minimal completely hemolytic amount of physiological salt solution) increases much more rapidly in the case of urea than it does with formaldehyde and in the latter case more rapidly than it does with boric acid.

The respective ratios for the three substances are:

	RATIO OF FINAL CONCENTRATION TO TREATING CONCENTRATION	
	Highest treating concentration	Lowest treating concentration
Boric acid	1–2	1–3
Formaldehyde	1–3.5	1–7.4
Urea	1–3.1	1–13.8

Differences in the osmotic effect of the three reagents corresponding with those already described were revealed in a comparative study of the quantitative relationships in the inhibition of the osmotic hemolysis with concentrated solutions of sodium chlorid and with solutions of the reagents themselves.

In these tests the treatment of the corpuscles and the determination of the minimal non-hemolytic concentrations of the various solutions was conducted in the manner of the tests

presented in table 7 of the previous paper. The osmotic concentrations tabulated under the heading of Δ were determined with the cryoscopic method.

The protocols of the tests are presented in tables 4, 5 and 6.

It is seen that when the corpuscles were treated with boric acid the osmotic concentrations of the minimal non-hemolytic concentrations of both sodium chlorid and boric acid are in each instance practically identical with the osmotic concentration of the treating mixture. This fact demonstrates that, given an equal intracorpuscular and extracorpuscular osmotic pressure, a

TABLE 4

Relation of the concentration of boric acid used in treatment to the minimal inhibiting concentrations of sodium chloride and boric acid

TREATING CONCENTRATION OF BORIC ACID			INHIBITING CONCENTRATION			
Amount of a 3.5 per cent solution in 1 cc. of original mixture	Per cent	Δ	NaCl		Boric acid in physiological salt solution	
			Per cent	Δ	Per cent	Δ
cc.						
0.5	1.75	1.115	1.85	1.095	1.54	1.07
0.45	1.575	1.075	1.75	1.05	1.40	1.00
0.4	1.4	1.00	1.62	0.97	1.275	0.95
0.35	1.225	0.94	1.5	0.91	1.05	0.88
0.3	1.05	0.88	1.4	0.85	0.945	0.84
0.25	0.875	0.835	1.3	0.83	0.77	0.80
0.2	0.7	0.78	1.18	0.74	0.595	0.75
0.15	0.525	0.73	1.08	0.69	0.42	0.67

slight lowering of the latter results in an appreciable hemolytic effect.

From the foregoing considerations it follows that if the assumption which we are examining is correct it should be expected that the hemolysis of the corpuscles which had been treated with any of the three different substances, in solutions of the same osmotic concentration, would be inhibited with the same minimal concentration of sodium chlorid.

Here again, however, the logical consequences of the theory were not fulfilled.

It is seen that when the osmotic concentration of the treating solution of boric acid was 1.00 the minimal inhibiting osmotic concentration of sodium chloride was 0.91, but when the corresponding osmotic concentration of formaldehyde (0.98) was used for treatment the minimal inhibiting osmotic concentration of sodium chlorid was only 0.747. Furthermore, it is seen that the minimal inhibiting osmotic concentration of sodium chlorid for corpuscles treated with urea in an osmotic concentration of 1.910 was less (1.00) than it was for corpuscles treated with formaldehyde of about the same osmotic concentration (1.868).

TABLE 5

Relation of the concentration of formaldehyde used in treatment to the minimal inhibiting concentrations of sodium chlorid, formaldehyde and boric acid

TREATING CONCENTRATION OF FORMALDEHYDE			INHIBITING CONCENTRATION					
Amount of a 4 per cent solution in 1 cc. of original mixture	Per cent	Δ	NaCl		Formaldehyde in physiological salt solution		Boric acid in physiological salt solution	
			Per cent	Δ	Per cent	Δ	Per cent	Δ
cc.								
0.5	2.0	2.035	2.1	1.26	1.6	1.7		
0.45	1.8	1.868	2.0	1.242	1.4	1.55		
0.4	1.6	1.70	1.8	1.082	1.24	1.4		
0.35	1.4	1.55	1.7	1.0	1.16	1.35	1.47	1.03
0.3	1.2	1.40	1.6	0.95	1.0	1.244	1.225	0.94
0.25	1.0	1.244	1.5	0.91	0.72	1.05	1.40	0.905
0.2	0.8	1.091	1.3	0.83	0.56	0.93	0.945	0.84
0.15	0.6	0.98	1.2	0.747	0.4	0.867	0.77	0.8

Similar discrepancies are disclosed in the quantitative results obtained when the treated corpuscles were tested with solutions of the three hemolytic reagents. For example, when the corpuscles had been treated with urea the osmotic concentrations of the minimal non-hemolytic concentrations of boric acid were always practically the same as those of sodium chlorid, but these were less than the corresponding ones of formaldehyde, the latter in turn, being less than those of urea. These discrepancies increase as the treating concentration of the urea increases.

In order to obtain a clear understanding of the significance of the results obtained with the corpuscles treated with formaldehyde and urea it is necessary first to outline the consequences of the tests which must have been anticipated on the basis of the assumption that osmotic pressure is a direct property of the solute.

As has been shown, the blood corpuscular membrane is perfectly permeable to all of the three hemolytic substances that we are studying.

TABLE 6

Relation of the concentration of urea used in treatment to the minimal inhibiting concentrations of sodium chlorid, urea, formaldehyde and boric acid

TREATING CONCENTRATION OF UREA			INHIBITING CONCENTRATION							
Amount of a 10 per cent solution in 1 cc. of original mixture	Per cent	Δ	NaCl		Urea in physiological salt solution		Formaldehyde in physiological salt solution		Boric acid in physiological salt solution	
			Per cent	Δ	Per cent	Δ	Per cent	Δ	Per cent	Δ
cc.										
0.5	5.0		1.95	1.19	3.5	1.78	1.4	1.55		
0.45	4.5		1.85	1.095	3.0	1.60	1.2	1.375		
0.4	4.0	1.91	1.7	1.0	2.5	1.40	1.0	1.244	1.47	1.03
0.35	3.5	1.78	1.6	0.95	2.25	1.35	0.8	1.091	1.225	0.94
0.3	3.0	1.60	1.45	0.90	1.8	1.2	0.6	0.95	1.085	0.905
0.25	2.5	1.40	1.35	0.835	1.5	1.05	0.4	0.869	0.77	0.8

It is evident, therefore, that, if osmotic pressure is directly exerted by the substance in solution, that pressure must be greatest at the instant of the sudden lowering of the extracorpuscular concentration, becoming rapidly less as more and more of the substance diffuses out of the cells into the medium of suspension.

Hence, in accordance with the theory that we are examining, a calculation of the counterpressure that should be expected to inhibit the osmotic hemolysis in any instance need take in consideration only by the original (treating) osmotic concentration of the hemolytic agent.

On the other hand, it is known that the corpuscular membrane is impermeable to sodium chlorid; hence the medium of suspension that contains only this substance should be considered as exerting a counter osmotic pressure which does not diminish but remains constant.

The incompatibility of the discrepancies which we have just been considering with the assumption that the solute exerts a direct osmotic pressure is the more clear, when it is recalled that in the case of boric acid only a slight lowering of the extracorpuscular concentration of that substance is sufficient to cause a perceptible hemolysis of the treated corpuscles. This observation stands in striking and significant contrast with the considerable lowering of the extracorpuscular concentration of urea which is necessary for the development of the slightest degree of destructive osmotic pressure by that substance.

This contrast is consistent with that already noted between the respective ratios of treating and final concentrations when the minimal completely hemolytic amount of physiological saline solution is added.

Reviewing the foregoing experiments we find:

1. That the same degree of osmotic hemolysis is not produced by identical "osmotic concentrations" of boric acid, formaldehyde and urea nor by a corresponding lowering in the concentration of the substances in the medium of suspension of the treated corpuscles.

2. That as the treating concentration of the three hemolytic substances is correspondingly diminished the ratio between that concentration and the final concentration, in the hemolytic experiment, increases disproportionately with the different substances.

3. That the osmotic hemolysis of corpuscles which have been treated with the three hemolytic substances in the same "osmotic concentration" is not inhibited by identical concentrations of sodium chloride nor of the hemolytic substances themselves.

4. That all of these facts contradict the assumption that osmotic pressure is exerted directly by the solute.

It will be seen that the obstacles presented by the foregoing experimental results to the acceptance of the theory of osmotic pressure which we have been considering, offer no hindrance to the adoption of the alternative view.

According to the latter conception the destructive force developed in osmotic hemolysis is exerted, not by the hemolytic substance itself, but by the water that diffuses *into* the corpuscles under the influence of the *higher concentration* of the hemolytic substance *within* the cells.

Under this conception another factor, in addition to that of the concentration of the hemolytic agent, must be taken into account; namely, the factor of *time*. The diffusion of water through a semipermeable membrane into a concentrated solution takes place not merely in the first moment of the process of osmosis but over a period of time.

Hence, it is evident that in the case of a diffusible solute, such as the three hemolytic agents employed above, the degree of destructive pressure developed within the corpuscles, under the alternative theory, need not be determined solely by the *original concentration* of the substance in the cells. The degree of that pressure could conceivably be dependent on the *length of time* during which an effective inequality of concentration within and without the cells is maintained.

In other words if one of the hemolytic substances diffuses out of the corpuscles more rapidly than the others the higher intracorpuscular concentration of that substance (upon which the inward diffusion of water depends) is consequently maintained for a shorter time than in the latter two cases and the resulting intracorpuscular pressure must, therefore, be less. Hence in order to explain the foregoing quantitative differences in the hemolytic effect of identical "osmotic concentrations" of the three hemolytic agents it is necessary only to assume that formaldehyde diffuses through the corpuscular membrane more rapidly than does boric acid and less rapidly than does urea.

For example, if formaldehyde diffuses out of the corpuscles more rapidly than does boric acid then a higher original "os-

motic concentration" of the former substance must be used in order to maintain an effective intracorpuscular concentration over the *period of time* necessary for the inward diffusion of a *hemolytic volume of water*. A similar explanation is applicable to the quantitative differences observed between the hemolytic action of formaldehyde and of urea.

The application of this explanation to the other consistent differences in the behaviour of the three hemolytic substances that have been described above is too obvious to require detailed analysis.

There remains, however, to subject the assumption on which the explanation is based to experimental examination. In other words, it has yet to be shown that actually formaldehyde diffuses through the corpuscular membrane more rapidly than boric acid and less rapidly than urea.

This question was investigated in the following manner:

The unit of corpuscles was suspended in 1 cc. of solutions of three reagents in such concentrations that in each case, after an equality of concentration inside and outside the corpuscles had been established the addition of 1 cc. of physiological salt solution would cause very strong hemolysis and the addition of 2 cc. would cause complete hemolysis. With the use of numerous identical mixtures tests could be made at different intervals of time, so that the length of time required for the equalization of the intracorpuscular and extracorpuscular concentration; that is, for the whole process of diffusion of the substance into the corpuscles, could be determined for each reagent.

The protocol of this experiment is presented in table 7.

It is seen that under the conditions of the test the diffusion time of boric acid was ninety seconds, that of formaldehyde was thirty seconds, while that of urea was too short—less than five seconds—to be accurately measured.

The results of this experiment, thus, confirm our assumption and support the conclusion that osmotic pressure is not a direct property of a solute but is solely the pressure exerted by water which has passed, by the yet unexplained process of diffusion,

TABLE 7

Determination of the diffusion time of the three hemolytic substances

Treating mixtures

	cc.
Boric acid (3.5 per cent)	0.4
Physiological salt solution	0.55
Blood suspension	0.05
Formaldehyde (4 per cent)	0.4
Physiological salt solution	0.55
Blood suspension	0.05
Urea (10 per cent)	0.8
Physiological salt solution	0.15
Blood suspension	0.05

	PERIOD OF CONTACT	HEMOLYSIS AFTER THE ADDITION OF PHYSIOLOGICAL SALT SOLUTION	
		1 cc.	2 cc.
Boric acid	5 seconds	0	0
	15 seconds	tr	w
	30 seconds	w	st
	1 minute	st	al.c
	1.5 minutes	v.st	c
	7 minutes	v.st	c
Formaldehyde	5 seconds	tr	w
	15 seconds	w	v.st
	30 seconds	v.st	c
	1 minute	v.st	c
	7 minutes	v.st	c
Urea	5 seconds	v.st	c
	10 seconds	v.st	c
	1 minute	v.st	c
	7 minutes	v.st	c

through a semipermeable membrane to the side of the higher osmotic concentration.

In further elucidation of this conception it may be said that there appears to be no more reason to assume that the water which diffuses into the corpuscles exerts an inward pressure during that process than there is for the now untenable theory of a direct outward osmotic pressure on the part of the solute. Such an assumption would leave without explanation the resulting hemolysis, which is caused by the increased internal pressure and which could not occur if, as is thought by some, the water diffuses through the membrane *under pressure* and if such diffusion ceases only when the internal pressure becomes so great as to equalize the inward pressure of the entering water.

ADDENDUM

In view of the differences in the rate of diffusion of the three hemolytic agents it could be anticipated that when corpuscles treated with a substance of a lesser rate of diffusion are placed in solutions of a substance of a higher rate of diffusion the equalization of the intra and extra-corpuscular concentration of the latter substance would be accomplished before all of the substance, used in treating the corpuscles, had diffused out of them. Under these circumstances a certain degree of hemolysis should, therefore, be expected, and the experiment conducted in this manner confirmed the anticipation.

The protocol of this experiment is presented in table 8.

It is seen that in every case the solutions of a substance of lower diffusion rate inhibited the hemolysis of corpuscles treated with a substance of higher diffusion rate, but that, on the other hand, solutions of a substance of higher diffusion rate could not inhibit the hemolysis of corpuscles treated with a substance of lower diffusion rate. It was found, furthermore, that no concentration of urea was able to inhibit, in the least degree, the hemolysis of corpuscles treated with boric acid.

In conclusion I wish to express my indebtedness to Dr. A. F. Coca for his kind advice and direction in carrying out these experiments.

TABLE 8

Showing the inhibiting effect of "iso-osmotic" solutions of boric acid, formaldehyde and urea upon the hemolysis of corpuscles treated with those substances

Treating mixtures

	cc.
Boric acid solution (3.5 per cent)	0.5
Physiological salt solutions	0.45
Blood suspension	0.05
Formaldehyde solution (4.0 per cent)	0.5
Physiological salt solution	0.45
Blood suspension	0.05
Urea solution (10 per cent)	0.75
Physiological salt solution	0.2
Blood suspension	0.05

TREATING	ADDING (1 cc.)			
	1.8 per cent solution of ClNa $\Delta=1.082$	Boric acid in physiological salt solution, 1.56 per cent, $\Delta=1.078$	Formaldeyde in physiological salt solution, 0.8 per cent, $\Delta=1.091$	Urea in physiological salt solution, 1.6 per cent $\Delta=1.09$
Boric acid	0		st	c
Formaldehyde	0	0		st
Urea	0	0	0	

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EXPERIMENTS ON THE EFFECT OF AGGLUTININS

B. FUJIMOTO

From the Forensic-Medical Institute of the Imperial University at Tokyo

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INTRODUCTION

As to the part which the agglutinins play in immunity we have yet little evidence. These bodies were formerly regarded as possessing a protective and curative power, but the bacteria that have been acted upon by them are apparently not altered in appearance and are as virulent as before agglutination. They can even grow in a specific agglutinating serum. Much of the work on this question tends, therefore, to show that they are not indicators of existing immunity. At present the agglutinins are generally regarded as playing a subsidiary rôle in immunity and as an aid to bacteriolysis and phagocytosis.

The following studies were undertaken in our laboratory in order to see whether agglutinins have any influence (1) on the glycolytic action of bacteria, (2) on the permeability of bacteria and red blood corpuscles for glucose solution and (3) on the resistence of red blood corpuscles for salt solution.

I. THE EFFECT OF THE AGGLUTININ ON THE GLYCOLYTIC ACTION OF BACILLUS COLI

As *Bacillus coli* as antigen can produce only agglutinin, we have preferred it to those bacteria, which, as antigen, produce bacteriolysin at the same time. The agglutinin used was obtained by immunizing rabbits with *Bacillus coli*. It agglutinated the homologous strain in a dilution of 1 : 4,000. The emulsion of the bacilli was prepared by adding normal salt solution to twenty-hour cultures on agar slants, the bacteria being carefully washed several times. To these emulsions was always

added a dose of agglutinin that was ten times as great as the amount called for by its titer. Then the mixtures were incubated at 37°C. and after they had stood for half an hour, the bacterial sediment was carefully washed, and salt solution was added to it up to 20 cc. Finally, there were added to these agglutinated emulsions 5 cc. of the glucose solution, which was so prepared that the emulsions contained 0.03 to 0.05 per cent of dextrose (extra pure). These emulsions were placed at 37°C. or at room temperature or in the ice chest. The results were noted after two or forty-eight hours. The Pavy-Kumagawa-Sudo method, which has been further modified by G. Momose, was employed for the estimations of sugar.

a. Tests with living bacilli

The first experiment was designed to show whether some of the functions of the living bacilli might be, at least, weakened by agglutinin, though the organisms are not killed by it. The results of the experiment are shown in table 1.

From table 1, it is seen that the glycolytic action of the living *Bacillus coli* was markedly weakened by agglutinin, when the emulsions were placed in the incubator or at room temperature. But when the emulsions were placed in the ice chest, contrary results were obtained; i.e., the glycolysis in the agglutinated emulsions in the ice chest takes place in a higher degree than that in the control emulsions. How may this be explained?

The glycolytic action of *Bacillus coli* depends upon two ferments: an endoenzyme and an exoenzyme. The production of the exoenzyme is considerably influenced by the temperature. The living bacteria can produce considerable exoenzyme at 37°C. or at room temperature, while in the ice chest this production decreases to its minimum. The glycolysis by exoenzyme in the ice chest is accordingly less than that at 37°C., as table 1 shows. On the other hand the endoenzyme cannot act so long as it is in the body of bacteria; but if the membrane of the bacteria is made permeable for it or their

TABLE 1

Effect of the agglutinin on the glycolytic action of living Bacillus coli

Experiment number			Dextrose	
			Agglutinated	Control
			per cent	*per cent*
I	At 37°C.	Initial amount (each ½ agar slant)	0.0473	0.0470
		After 2½ hours	0.0460	0.0290
		After 5 hours	0.0377	0.0224
II	At 37°C.	Initial amount (each ½ agar slant)	0.0286	0.0289
		After 3 hours	0.0246	0.0134
		After 5 hours	0.0194	0.0052
III	In ice chest	Initial amount (each 2 agar slants)	0.0381	0.0390
		After 24 hours	0.0303	0.0393
		After 48 hours	0.0220	0.0378
		After 72 hours	0.0056	0.0201
IV	At 37°C.	Initial amount (each ¼ agar slant)	0.0486	0.0478
		After 3 hours	0.0397	0.0334
		After 5 hours	0.0356	0.0284
	At room temperature (August)	After 3 hours	0.0450	0.0478
		After 5 hours	0.0399	0.0348
	In ice chest	After 3 hours	0.0480	0.0478
		After 5 hours	0.0472	0.0480
		After 48 hours	0.0227	0.0387
		After 72 hours	0.0103	0.0184
V	At 37°C.	Initial amount (each 1 agar slant)	0.0384	0.0378
		After 2 hours	0.0332	0.0263
		After 4 hours	0.0279	0.0210
	In ice chest	After 2 hours	0.0377	0.0381
		After 4 hours	0.0370	0.0379
		After 24 hours	0.0278	0.0316
		After 48 hours	0.0102	0.0284

body is destroyed, then it diffuses into the medium of suspension and can exert its glycolytic effect. The question why the glycolysis in the agglutinated emulsion in the ice chest is

greater than that in the not agglutinated one can be solved only by assuming that the body of the agglutinated bacteria was so altered by agglutinin that their endoenzyme is made free from their body by it. Of course, the glycolysis produced with the agglutinated emulsion at 37°C. by the endoenzyme should, also, be greater than that produced with the non-agglutinated one; but, as the decrease of the production of exoenzyme due to the agglutinin is far greater than the increase of the endoenzyme due to that agent, the glycolysis with the agglutinated emulsion at 37°C, is actually less than that with the non-agglutinated one. (see table 1). We could prove in the following experiments that this assumption was true.

b. Tests with dead bacilli

As living bacilli can produce glycolytic ferment (exoenzyme), the bacilli were previously killed with chloroform or by heating at 58°C., in order to eliminate this factor. The results are shown in table 2.

In order to see whether the heated emulsions and those treated with chloroform were sterile, several portions were taken from them on agar slants and incubated. The emulsions, which were treated with chloroform, were found to be sterile, while those, which had been heated at 58°C. for two hours, once daily for three days, were not completely sterile. However, higher temperatures were avoided, because of the danger of weakening the ferments (see table 2, experiment 1).

As we see from experiment 5 in table 2 the glycolysis with the agglutinated emulsions increased the more they were heated at 58°C. The glycolysis in those emulsions, which were heated only once at 58°C. for two hours, was less than that in the control emulsions, because the bacilli mostly survived. But those, which were heated twice, were half killed; hence the glycolysis with them was equal to that of the control. As nearly all of the bacteria were killed by the third heating, the glycolysis with thrice heated emulsions was greater than that with the control emulsion; in this case, the glycolysis caused by the endoenzyme has overcome that caused by exoenzyme.

TABLE 2

Effect of agglutinin on the dead Bacillus coli

EXPERIMENT NUMBER				DEXTROSE	
				Agglutinated	Not agglutinated
				per cent	*per cent*
I	Killed by heating at 100°C. for 5 minutes	At 37°C.	Initial amount (each 2 agar slants)	0.0356	0.0351
			After 24 hours	0.0348	0.0364
		At room temperature. (July)	After 24 hours	0.0352	0.0357
II	Heated at 58°C. for 2½ hours	At 37°C.	Initial amount (each 1 agar slant)	0.0471	0.0475
			After 24 hours	0.0346	0.0056
		At room temperature) (July) in ice chest	After 24 hours	0.0346	0.0080
			After 24 hours	0.0354	0.0434
			After 72 hours	0.0252	0.0256
III	Killed with chloroform for 20 hours	At 37°C.	Initial amount (each ⅓ agar slant	0.0384	0.0384
			After 6 hours	0.0252	0.0336
		In ice chest	After 24 hours	0.0270	0.0362
			After 48 hours	0.0187	0.0296
IV	Heated at 58°C. for 2 hours, once daily for 3 days	At 37°C.	Initial amount (each ⅓ agar slant	0.0307	0.0305
			After 4 hours	0.2057	0.0290
			After 24 hours	0.0095	0.0305
V	Heated at 58°C. for 2 hours once	At 37°C.	Initial amount (each ½ agar slant)	0.0458	0.0455
			After 5 hours	0.0372	0.0358
	Twice	At 37°C.	After 5 hours	0.0312	0.0308
	Thrice	At 37°C.	After 5 hours	0.0280	0.0339

TABLE 2—*Continued*

EXPERIMENT NUMBER				DEXTROSE	
				Agglutinated	Not agglutinated
				per cent	*per cent*
VI	Treated with tannin solution	At 37°C.	Initial amount (each ½ agar slant)	0.0288	0.0290
			After 3 hours	0.0278	0.0134
			After 5 hours	0.0252	0.0052
VII	Triturated carefully	At 37°C.	Initial amount (each ½ agar slant)	0.0288	0.0290
			After 3 hours	0.0186	0.0134
			After 5 hours	0.0118	0.0052

The glycolytic action of *Bacillus coli* may naturally be weakened by some other treatments which are harmful to them. We have treated the *Bacillus coli* with a 1 per cent solution of tannic acid, which can agglutinate them as does agglutinin. The glycolytic action of bacteria treated in such a way was examined in a similar manner. The glycolysis in those bacteria, which were simply triturated, were also examined. The results are shown in the foregoing table 2, experiment 6.

Conclusion. The glycolytic action of the living *Bacilli coli* is weakened markedly by agglutinin, and the agglutinin is able to injure the membrane of bacilli in some degree.

II. THE EFFECT OF THE AGGLUTININS ON THE PERMEABILITY OF BACTERIA AND RED BLOOD CORPUSCLES FOR GLUCOSE

In the foregoing experiments it was proved that the agglutinin may influence the glycolytic function of bacteria. Several experiments will now be presented, which were performed in order to see whether the permeability of bacteria and red blood corpuscles for glucose is changed. The hemagglutinin was obtained by immunizing rabbits with the washed red blood corpuscles of fowl and it was of high titer (1:8,000)). The

serum was always used in an amount 20 times that indicated by its titer. To 20 cc. of the emulsions were added 5 cc. of physiological salt solution, which contained a certain quantity of glucose, and the emulsions were immediately placed in the ice chest because the blood of fowl decomposes very easily. The results are shown in table 3.

TABLE 3

			CONCENTRATION OF BLOOD EMULSION		
			40 per cent	20 per cent	10 per cent
I	Sensitized with hemagglutinin	Initial amount		0.0251	0.0240
		After 20 hours		0.0232	0.0243
	Not sensitized	Initial amount		0.0258	0.0253
		After 20 hours		0.0254	0.0249
II	Sensitized with hemagglutinin	Initial amount	0.0384	0.0388	
		After 5 hours (at room temperature)	0.0381	0.0388	
	Not sensitized	Initial amount	0.0391	0.0378	
		After 5 hours (at room temperature)	0.0384	0.0380	
III	Sheep's blood sensitized with hemolysin	Initial amount		0.0242	0.0242
		After 20 hours		0.0243	0.0241
	Not sensitized	Initial amount		0.0240	
		After 20 hours		0.0250	

As we see from the table 3, the permeability of the red blood corpuscles is not altered at all by hemagglutinin. The permeability of the membrane of *Bacillus coli* was examined in the same manner. The glycolytic action of the bacteria was avoided by heating them at 100°C. or by placing them in the ice chest. The results of this experiment are presented in table 4.

We could, thus, find no change in the permeability of bacteria.

TABLE 4

		DEXTROSE IN	
		Agglutinated	Not agglutinated
		per cent	*per cent*
Killed at 100°C. (each 2 agar slants)	Initial amount	0.0356	0.0352
	After 20 hours (at room temperature)	0.0348	0.0364
Living bacteria (each 2 agar slants)	Initial amount	0.0342	0.0338
	After 6 hours (in ice chest)	0.0340	0.0341
Living bacteria (each 1 agar slant)	Initial amount	0.0384	0.0384
	After 2 hours	0.0377	0.0381
	After 4 hours	0.0370	0.0379

III. THE EFFECT OF HEMAGGLUTININ ON THE RESISTANCE OF RED BLOOD CORPUSCLES FOR HYPOTONIC SOLUTIONS

We have also investigated the resistance of the agglutinated blood corpuscles to hypotonic salt solutions. Friedberger and Rössle (4) demonstrated that the resistance of red blood corpuscles, acted upon by hemolysin, was not weakened, but rather strengthened. Von Dungern and Coca (5) came to the same conclusion as to the resistance of sensitized corpuscles for solutions of sodium oleate.

The washed blood corpuscles of fowl were suspended in 0.5 per cent saline solution. One cubic centimeter of this emulsion was placed in test tubes which contained 1 cc. of saline solution of different dilutions. The resulting hemolysis was noted after fifteen minutes. In the following table $\times$ indicates complete hemolysis.

The resistance of the blood corpuscles to hypotonic saline solution may be slightly decreased by agglutinin or hemolysin, as the above table shows.

TABLE 5

	CONCENTRATION OF THE SALINE SOLUTION	SENSITIZED		NOT SENSITIZED
	per cent			
Hemagglutinin (Blood corpuscles of fowl)	0.31	+		+
	0.32	+		±
	0.33	+		±
	0.34	+		±
	0.35	±		−
	0.36	±		−
	0.37	−		−
		Sensitized with hemolysin as to its titer		
		25 times	2 times	
Hemolysin (Blood corpuscles of sheep)	0.54	+	+	+
	0.55	+	±	±
	0.56	±	±	±
	0.57	±	±	±
	0.58	±	±	±
	0.59	±	−	−
	0.60	−	−	−

CONCLUSIONS

1. The glycolytic action of the living *Bacillus coli* may be markedly weakened by agglutinin.

2. The agglutinin is able so to change the cell membrane of the bacteria, that their endoenzyme can permeate it.

3. Neither agglutinin nor hemagglutinin alters the permeability of bacteria and red blood corpuscles for glucose.

4. The resistance of the blood corpuscles to hypotonic solutions is slightly decreased by agglutinin or hemolysin.

I wish to express my thanks to Prof. Dr. K. Katayama and Prof. Dr. S. Mita for suggesting this problem and for their kind advice.

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A COMPARATIVE STUDY OF HEMOLYTIC COMPLEMENT AND ANTIBODIES IN OXALATED PLASMA AND SERUM

SUSUMU WATANABE

From the McManes Laboratory of Experimental Pathology of the University of Pennsylvania

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Whether or not complement exists preformed in the circulating plasma or develops upon coagulation of the blood and the production of serum, has been for many years a debatable subject between the adherents of the humoral and cellular theories of immunity.

Gengou (1) in 1901 concluded from his studies with plasma secured at a low temperature, that complement or alexin is not present in the circulating blood, but appears only after injury to the leucocytes during the process of coagulation. These results were apparently confirmed by Herman (2) and later Gurd (3) in an excellent study of this subject, concluded that complement does not exist as such in the circulating plasma but as "complementogen," which is rendered active by a substance analogous to thrombokinase, liberated by the leucocytes.

Numerous other investigations however, as those by Dömeny (4), Sweet (5), Hewlett (6), Löwit and Schwarz (7), Lambotte (8), Hoessli (9), von Dungern (10), Jouan and Staub (11) and Addis (12) have tended to disprove the original findings of Gengou and to show that plasma contains as much complement as serum. Falloise (13) and Schneider (14) for example, employing Gengou's methods, obtained results diametrically opposite.

Few investigations have been devoted to the subject of antibodies in plasma; Dreyer and Walker (15) have reported the presence of larger amounts of agglutinin for *B. typhosus* in rab-

bit plasma than in serum and Cowie (16) found more opsonin for staphylococcus aureus in the human plasma of the placental blood than in the serum. Otani (17) observed a greater degree of phagocytosis in the citrated plasma of tuberculous persons than in that of normal persons. Of further interest in this connection may be mentioned the investigation of Gessard (18) showing that the plasma of the cow contains more antityrosinase than the serum and the work of Blaziot (19) indicating that oxalated plasma neutralizes the toxicity of tissue extracts more effectually than serum.

With this diversity in results and opinions regarding the presence of complement in plasma and the desirability of further investigations not only in this subject but also regarding the presence of antibodies in plasma, Professor Kolmer suggested that I undertake a study of these problems after the following plan:

1. To study the various methods described for the collection of plasma with the idea of evolving an acceptable technic. This phase of the problem was first approached and resulted in my devising a method which has proven quite satisfactory and capable of yielding uniform and acceptable plasmas in the majority of trials.

2. To determine by processes of titration whether hemolytic complement, natural thermolabile and thermostabile antisheep hemolysins and natural typhoid agglutinin exist in the plasmas of normal persons as compared with the corresponding sera.

3. To ascertain whether syphilis antibody concerned in the Wassermann reaction exists in plasmas of luetic persons.

4. To determine whether hemolytic complement, natural antisheep hemolysin and hemagglutinin exist in the plasmas and sera of normal rabbits; also natural typhoid agglutinin and the non-specific complement fixing substances for bacterial and syphilis antigens described by Professor Kolmer and his associates (20, 21).

5. To immunize rabbits with sheep erythrocytes and test the plasmas and sera by titrations for immune antisheep hemolysin and hemagglutinin.

6. To immunize rabbits with *B. tyhosus* and titrate the plasmas and sera for the presence of immune typhoid agglutinin and complement fixing antibodies.

In all experiments the tests with each plasma and serum were strictly comparative and conducted at the same time, with the same reagents and in the same manner.

A METHOD FOR SECURING PLASMA

For the collection of plasma various anticoagulants have been employed, namely, magnesium sulphate, sodium chlorid, mono potassium phosphate, sodium oxalate, sodium fluorid, potassium citrate, iron sulphate, pepton, histon, soap and leech extract; some of these as pepton, leech extract and iron sulphate have been injected intravenously to lower the coagulation time of the blood.

The large amounts of sodium chlorid, magnesium sulphate and mono potassium phosphate required for the prevention of coagulation are objectionable by reason of their possible influence upon the complement and antibodies which may be present.

Sodium oxalate and sodium fluorid, on the other hand, inhibit coagulation in very small amounts by means of precipitation of the calcium of the blood and the prevention of thrombin production; but potassium citrate inhibits coagulation, as shown by Schmidt (22), by means of higher concentration of potassium citrate itself similar to the action of sodium chlorid, magnesium sulphate and other salts.

Of these anticoagulants sodium oxalate and sodium fluorid appear to be free of injurious influence upon the antibodies which may be present and they have been judged best for our studies, the former being employed routinely.

In order to avoid dilution of the plasma and the possibility of error due to the presence of anticoagulating fluids, it was necessary to avoid fluids in securing plasma.

In the paraffiin tube method devised by Freund, centrifuge tubes were coated with paraffin and chilled to a low tempera-

ture in order to prevent coagulation and the collection of plasma. In my experience the method proved unsatisfactory inasmuch as the plasma regularly coagulated when brought to body temperature.

Prof. George H. Meeker then very kindly taught me a technic devised and used by him with success in the collection of plasma consisting in marking an appropriate centrifuge tube at 30 cc. and placing within 0.75 cc. of a 2 per cent solution of sodium oxalate following by drying in a gas flame with even distribution of the oxalate up to the fiduciary mark, but avoiding boiling.

Professor Meeker found that 0.0005 gram sodium oxalate was sufficient to decalcify 1 cc. of human blood. Blood was then collected from a congested vein up to the 30 cc. mark followed by admixing with the powdered oxalate adhering to the walls of the centrifuge tube and immediate centrifugalization.

The principle of this method is excellent and I succeeded with it in obtaining plasma at times but it was not always possible to avoid having blood come in contact with the unprotected glass above the mark or to secure even mixture of blood and oxalate; for these reasons partial coagulation not infrequently occurred with the production of serum.

These faults were corrected in the following method which I devised as a combination of the paraffin tube and Meeker methods:

1. A centrifuge tube is marked to indicate a volume of 20 cc. and 1 cc. of a 2 per cent solution of sodium oxalate added; this amount of oxalate is double that used by Meeker and effectually presents coagulation.

2. The solution of oxalate is then dried as evenly as possible in the test tube held over a gas flame up to and a little beyond the 20 cc. mark.

3. The upper portion of the tube is now coated with a thin layer of molden paraffin by means of a small soft brush so that there is no unprotected glass.

4. Blood is collected by means of a short, wide bored needle into the prepared tube, which is gently rotated during and immediately after the collection of 20 cc. and immediately centri-

fuged for thirty minutes at high speed. The supernatant plasma is then pipetted to a second plain centrifuge tube and centrifuged at high speed for two to two and one-half hours. The resulting plasma is free of leucocytes and almost or entirely free of blood platelets.

For the purpose of this investigation an additional 20 cc. of blood were collected in a plain centrifuge tube, allowed to stand at room temperature for several hours and centrifuged for the serum, both plasma and serum being ready for testing at the same time.

INFLUENCE OF TECHNIC EMPLOYED UPON THE PROPERTIES OF PLASMA AND SERUM

According to this technic, however, plasma is subjected to three influences which serum escapes, namely, (1) the presence of oxalate, (2) a longer period of centrifugalization and (3) the heat engendered by the process of centrifuging. In order to determine the influence of these factors certain preliminary tests were conducted as follows:

1. Influence of sodium oxalate upon complement

Guinea-pigs were bled in plain tubes and after coagulation 20 cc. of blood were transferred to our regular oxalate-paraffin tube, well broken up and centrifuged after standing at room temperature for three hours. The balance of untreated clot was broken up and centrifuged at the same time followed by a titration of both portions for hemolytic complement with an antisheep system. The results of an experiment are shown in table 1 and indicate that the amount of oxalate employed has but very slight and almost negligible influence upon complement activity.

The results of similar experiments conducted with sheep serum for hemolytic activity for guinea-pig and rabbit erythrocytes due to the presence of natural hemolysins for these cells in addition to complement, are shown in table 2 and indicate that sodium oxalate in the amount employed in our method has practically no influence upon the complement.

TABLE 1

*Titration of hemolytic complement of guinea-pig sera with and without addition of sodium oxalate**

COMPLEMENT 1:20	ANTI-SHEEP HEMOLYSIN	2.5 PER CENT SHEEP CELLS	0.85 PER CENT SALT SOLUTION	NO SODIUM OXALATE	WITH SODIUM OXALATE
cc.	*units*	*cc.*	*cc.*		
0.3	2	1.0	0.5	M.H†	M.H
0.35	2	1.0	0.45	C.H	V.M.H
0.4	2	1.0	0.4	C.H	C.H
0.45	2	1.0	0.35	C.H	C.H
0.5	2	1.0	0.3	C.H	C.H
0.6	2	1.0	0.2	C.H	C.H
0.7	2	1.0	0.1	C.H	C.H

* Water bath incubation for one hour.

† C.H indicates complete hemolysis; V.M.H = very marked hemolysis; M.H = marked hemolysis.

TABLE 2

*Titration of hemolytic activity of sheep sera for guinea-pig and rabbit erythrocytes with and without addition of sodium oxalate**

UNDILUTED SERUM	GUINEA-PIG CELLS			RABBIT CELLS		
	Undiluted cells	No sodium oxalate	With sodium oxalate	Undiluted cells	No sodium oxalate	With sodium oxalate
cc.	*cc.*			*cc.*		
0.1	0.017	C.H†	C.H	0.001	C.H	C.H
0.1	0.018	C.H	C.H	0.002	C.H	C.H
0.1	0.019	V.M.H	M.H	0.003	C.H	C.H
0.1	0.020	M.H	M.H	0.004	C.H	C.H
0.1	0.021	M.H	M.H	0.005	V.M.H	V.M.H
0.1	0.022	M.H	M.H	0.006	V.M.H	S.H
0.1	0.023	M.H	M.H	0.007	S.H	S.H
0.1	0.024	M.H	M.H	0.008	S.H	S.H
0.1	0.025	M.H	M.H	0.009	S.H	S.H

* Water bath incubation for one hour.

† C.H indicates complete hemolysis; V.M.H = very marked hemolysis; M.H = marked hemolysis; S.H = slight hemolysis.

Larger amounts of oxalate however, exert a very distinct inhibitory effect upon complement as shown in experiments consisting in adding 20 cc. of coagulated blood to tubes containing four times the usual amount of oxalate, namely, the dried oxalate secured by evaporation of 4 cc. of a 2 per cent solution,

followed by centrifuging in the usual manner. These results are shown in table 3.

Similar experiments with plasma collected with an excess of oxalate and plain serum titrated for total hemolytic activity for

TABLE 3

*Titration of complement in human serum containing an excess of sodium oxalate and in plain serum**

AMOUNTS OF SERUM 1:10	ANTI-SHEEP HEMOLYSIN	1 PER CENT SHEEP CELLS	0.85 PER CENT SALT SOLUTION	NO SODIUM OXALATE	WITH SODIUM OXALATE
cc.	*units*	*cc.*	*cc.*		
0.1	2	1.0	0.7	S.H†	S.H
0.2	2	1.0	0.6	C.H	S.H
0.3	2	1.0	0.5	C.H	S.H
0.4	2	1.0	0.4	C.H	S.H
0.5	2	1.0	0.3	C.H	M.H
0.6	2	1.0	0.2	M.H	M.H

* Water bath incubation for one hour.

† C.H indicates complete hemolysis; M.H = marked hemolysis; S.H = slight hemolysis.

TABLE 4

Titration of natural antisheep hemolysin and complement in human plasma containing a large excess of sodium oxalate and in plain serum

AMOUNT OF PLASMA AND SERUM	5 PER CENT SHEEP CELLS	0.85 PER CENT SALT SOLUTION	PLAIN SERUM	PLASMA WITH AN EXCESS OF OXALATE
cc.	*cc.*	*cc.*		
0.1	0.1	0.9	C. H*	S. H
0.1	0.2	0.8	C. H	S. H
0.1	0.3	0.7	C. H	S. H
0.1	0.4	0.6	C. H	S. H
0.1	0.5	0.5	C. H	S. H
0.1	0.6	0.4	C. H	S. H
0.1	0.7	0.3	C. H	N. H
0.1	0.8	0.2	C. H	N. H
0.1	0.9	0.1	M. H	N. H
0.1	1.0	0	M. H	N. H

* C. H indicates complete hemolysis; M. H = marked hemolysis; S. H = slight hemolysis; N. H = no hemolysis.

sheep cells due to the presence of natural antisheep hemolysin in addition to complement, yielded similar results as shown in table 4 and indicated the anticomplementary influence of an excessively large amount of oxalate.

2. Influence of centrifuging and higher temperature

With a laboratory temperature of 22°C. experiments have shown that water centrifuged for one hour had a temperature of 27°C.; at the end of two hours 31°C. and after three hours 33°C. Gurd (3) has stated that guinea-pig blood kept at body temperature yields complement serum of maximum activity whereas at low temperatures activity is decreased.

TABLE 5

Titration of complement in guinea-pig sera, exposed at different temperatures

A. The clot is placed immediately after drawing in incubator (37°C.) for three hours, broken up, centrifuged and the serum tested.

B. The clot is placed at room temperature (22°C.) for three hours, broken up, centrifuged and the serum tested.

C. The clot is broken up, centrifuged for three hours and the serum tested.

D. The clot is placed in refrigerator (2°C.) for three hours, then broken up, centrifuged and the serum tested.

COMPLEMENT SERUM 1:20	A	B	C	D
cc.				
0.1	S.H	S.H	S.H	S.H
0.2	M.H	M.H	M.H	M.H
0.3	V.M.H	M.H	V.M.H	M.H
0.4	C.H	C.H	C.H	V.M.H
0.5	C.H	C.H	C.H	V.M.H
0.6	C.H	C.H	C.H	C.H
0.7	C.H	C.H	C.H	C.H
0.8	C.H	C.H	C.H	C.H

In my experiments the blood of the same persons and guinea-pigs divided into equal parts and kept at 1° to 3°C., room and incubator temperature yielded sera of varying complement activity with a marked decrease at the low temperatures, but sera kept at room temperature, as were the sera used in comparative tests of serum and plasma, and centrifuged as were the plasmas, showed no variation in complement activity. The results of an experiment of this kind are shown in table 5.

TABLE 6

Titration of complement in human and guinea-pig serum, exposed at different temperatures and for different periods

A. The clot is broken up immediately after coagulation, centrifuged and serum tested.
B. The clot is placed an hour in incubator, broken up, centrifuged and serum tested.
C. The clot is exposed in room temperature for twenty-four hours, broken up, centrifuged and serum tested.
E. Same as C, but is placed in refrigerator.

AMOUNT OF COMPLEMENT	HUMAN SERUM 1:10, 1 CC. OF 1 PER CENT SHEEP CELLS				GUINEA-PIG SERUM 1:20, 1 CC. OF 2.5 PER CENT SHEEP CELLS							
					No. 1				No. 2			
	A	B	C	D	A	B	C	D	A	B	C	D
cc.												
0.1	N.H*	N.H	N.H	N.H	M.H	M.H	M.H	M.H	M.H	M.H	M.H	M.H
0.2	N.H	N.H	V.S.H	V.S.H	M.H	M.H	V.M.H	V.M.H	M.H	M.H	V.M.H	V.M.H
0.3	S.H	V.S.H	M.H	M.H	V.M.H	M.H	V.M.H	V.M.H	M.H	M.H	V.M.H	V.M.H
0.4	V.M.H	M.H	V.M.H	V.M.H	V.M.H	M.H	V.M.H	V.M.H	M.H	M.H	V.M.H	M.H
0.5	C.H	V.M.H	C.H	C.H	C.H	C.H	C.H	C.H	M.H	M.H	V.M.H	V.M.H
0.6	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	M.H	V.M.H	C.H	C.H
0.7	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H
0.8	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H

* C.H = complete hemolysis; V.M.H = very marked hemolysis; M.H = marked hemolysis; S.H = slight hemolysis; N.H = no hemolysis.

In this experiment an antisheep hemolytic system was employed with two units of hemolysin;[1] 1 cc. of a 2.5 per cent suspension of sheep cells and water bath incubation for an hour.

The results of a similar experiment conducted with human and guinea-pig complements titrated in an antihuman and antisheep hemolytic system are shown in table 6.

Additional experiments were conducted with human serum titrated for complement alone and for complement and natural antisheep hemolysin; the results of an experiment are shown in table 7.

TABLE 7

Titration of complement alone and natural hemolysin and complement of human serum, exposed at different temperatures

A. The clot is placed for thirty hours at room temperature, broken up, centrifuged and serum tested.

B. Same as A, but is placed in refrigerator.

TITRATION OF COMPLEMENT				TITRATION OF NATURAL HEMOLYSIN AND COMPLEMENT			
Serum 1:10	1 per cent sheep cells	A	B	Serum undiluted	5 per cent sheep cells	A	B
cc.	*cc.*			*cc.*	*cc.*		
0.1	1.0	S. H	N. H	0.1	0.1	C. H	N. H
0.2	1.0	V. M. H	N. H	0.1	0.2	C. H	N. H
0.3	1.0	V. M. H	N. H	0.1	0.3	C. H	N. H
0.4	1.0	V. M. H	N. H	0.1	0.4	V. M. H	N. H
0.5	1.0	C. H	N. H	0.1	0.5	V. M. H	N. H
0.6	1.0	C. H	N. H	0.1	0.6	M. H	N. H
				0.1	0.7	M. H	N. H
				0.1	0.8	M. H	N. H
				0.1	0.9	M. H	N. H
				0.1	1.0	M. H	N. H

The results of these experiments have shown:

1. Bloods broken up and centrifuged soon after bleeding or kept for one to three hours at room or incubator temperature for three hours followed by centrifuging, yielded complements of approximately equal hemolytic activity.

[1] A unit of hemolysin was the smallest amount of rabbit antisheep hemolysin producing complete hemolysis of 1 cc. of 2.5 per cent suspension sheep cells with 0.05 cc. guinea-pig complement in one hour.

2. Bloods kept in the refrigerator at a low temperature yielded complements of much reduced hemolytic activity.

3. Centrifuging plasma for three hours during which the temperature was raised to 33°C. does not have any appreciable influence upon the hemolytic activity of the complement present.

CONCERNING THE PRESENCE OF HEMOLYTIC COMPLEMENT IN HUMAN PLASMA

With these technical matters settled the main tests with plasma and serum were conducted. For the purpose of studying the plasmas of human individuals, the bloods of eighteen persons were secured from among patients giving consent in the skin clinic of Dr. Jay F. Schamberg, plasma and serum being secured from each person at the same time.

Technic. Both plasma and serum were diluted 1:10 with sterile physiological saline solution and titrated in amounts of 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 cc. with two units of antisheep hemolysin and 1 cc. of a 1 per cent suspension of sheep cells with water bath incubation for one hour followed by placing the tubes in the refrigerator over night and recording the results the following morning. With the addition of sheep cells to the plasmas coagulation frequently occurred in the water bath but in a degree not usually sufficient to interfere with hemolysis.

Results. The results of these titrations are shown in table 8.

1. Of the blood of eighteen persons studied, the plasmas of eleven contained slightly more complement than the sera; seven of the plasmas contained an amount of complement equal to the serum.

2. The average unit of complement in the undiluted plasmas was 0.0312 cc. and of the sera 0.0412 cc.

CONCERNING NATURAL ANTISHEEP HEMOLYSIN AND COMPLEMENT IN HUMAN PLASMA AND SERUM

As is well known over 90 per cent of active human sera contained natural antisheep hemolysin and it was considered of interest to determine whether or not this natural or normal antibody was to be found in plasma.

TABLE 8

Titration of complement in human plasmas and sera

NUMBER	INITIAL OF PERSONS	SUBSTANCE 1:10	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.5 cc.	0.6 cc.
1	K	Plasma	N.H*	S.H	M.H	V.M.H	C.H	C.H
		Serum	N.H	S.H	M.H	V.M.H	C.H	C.H
2	S	Plasma	N.H	V.S.H	S.H	M.H	C.H	C.H
		Serum	N.H	V.S.H	S.H	M.H	C.H	C.H
3	W	Plasma	N.H	S.H	M.H	C.H	C.H	C.H
		Serum	N.H	S.H	M.H	M.H	C.H	C.H
4	G	Plasma	N.H	V.S.H	M.H	M.H	C.H	C.H
		Serum	N.H	N.H	N.H	N.H	N.H	N.H
5	M	Plasma	N.H	S.H	M.H	C.H	C.H	C.H
		Serum	N.H	V.S.H	M.H	M.H	C.H	C.H
6	D	Plasma	N.H	M.H	C.H	C.H	C.H	C.H
		Serum	N.H	M.H	C.H	C.H	C.H	C.H
7	R	Plasma	M.H	M.H	C.H	C.H	C.H	C.H
		Serum	N.H	M.H	C.H	C.H	C.H	C.H
8	S	Plasma	N.H	C.H	C.H	C.H	C.H	C.H
		Serum	V.S.H	C.H	C.H	C.H	C.H	C.H
9	J	Plasma	M.H	C.H	C.H	C.H	C.H	C.H
		Serum	V.S.H	M.H	M.H	M.H	C.H	C.H
10	S	Plasma	N.H	C.H	C.H	C.H	C.H	C.H
		Serum	N.H	M.H	M.H	C.H	C.H	C.H
11	G	Plasma	N.H	M.H	C.H	C.H	C.H	C.H
		Serum	N.H	M.H	M.H	C.H	C.H	C.H
12	C	Plasma	N.H	S.H	M.H	C.H	C.H	C.H
		Serum	N.H	N.H	S.H	V.M.H	C.H	C.H
13	T	Plasma	M.H	C.H	C.H	C.H	C.H	C.H
		Serum	M.H	C.H	C.H	C.H	C.H	C.H

TABLE 8—*Continued*

NUMBER	INITIAL OF PERSONS	SUBSTANCE 1:10	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.5 cc.	0.6 cc.
14	K	Plasma	S.H	C.H	C.H	C.H	C.H	C.H
		Serum	N.H	M.H	C.H	C.H	C.H	C.H
15	F	Plasma	S.H	C.H	C.H	C.H	C.H	C.H
		Serum	N.H	N.H	N.H	V.S.H	S.H	M.H
16	H	Plasma	S.H	C.H	C.H	C.H	C.H	C.H
		Serum	S.H	C.H	C.H	C.H	C.H	C.H
17	W	Plasma	N.H	V.M.H	C.H	C.H	C.H	C.H
		Serum	N.H	V.M.H	C.H	C.H	C.H	C.H
18	I	Plasma	N.H	V.M.H	C.H	C.H	C.H	C.H
		Serum	N.H	V.M.H	C.H	C.H	C.H	C.H

* C.H = complete hemolysis; V.M.H = very marked hemolysis; M.H = marked hemolysis; S.H = slight hemolysis; V.S.H = very slight hemolysis; N.H = no hemolysis.

Technic. The plasmas and sera of the same eighteen persons referred to above were titrated as follows: into a series of ten test tubes were placed increasing doses of 2.5 or 5 per cent suspensions of washed sheep cells varying from 0.1 to 1 cc.; into each tube was then placed 0.1 cc. of plasma or serum and sufficient salt solution to make 2 cc. followed by mixing, water bath incubation for one hour and readings the following morning. These reactions expressed the total hemolytic activity for sheep cells of each plasma and serum and are shown in table 9.

The results may be summarized as follows:

1. The total hemolytic activity of seven plasmas was greater than the corresponding sera, weaker with two and equal in nine.

2. When the total amounts of corpuscle suspension hemolysed by the total amounts of plasmas and sera were computed, it was found that the plasmas hemolysed 11.7 cc. of corpuscle suspension as against 10.3 cc. by the sera.

TABLE 9

Titration of normal antisheep hemolysin and complement in human plasmas and sera

NUMBER	2.5 PER CENT SHEEP CELLS	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.5 cc.	0.6 cc.	0.7 cc.	0.8 cc.	0.9 cc.	1.0 cc.
1	Plasma	N.H	N.H	N.H	N.H	N.H	N.H	N.H	N.H	N.H	N.H
	Serum	N.H	N.H	N.H	N.H	N.H	N.H	N.H	N.H	N.H	N.H
2	Plasma	N.H	N.H	N.H	N.H	N.H	N.H	N.H	N.H	N.H	N.H
	Serum	C.H	M.H	M.H	M.H	M.H	M.H	M.H	M.H	M.H	M.H
3	Plasma	C.H	C.H	C.H	C.H	C.H	C.H	C.H	M.H	M.H	M.H
	Serum	C.H	C.H	C.H	C.H	C.H	M.H	M.H	M.H	M.H	M.H
4	Plasma	C.H	C.H	C.H	C.H	M.H	M.H	M.H	M.H	M.H	M.H
	Serum	N.H	N.H	N.H	N.H	N.H	N.H	N.H	N.H	N.H	N.H
5	Plasma	C.H	C.H	C.H	C.H	C.H	C.H	M.H	M.H	M.H	M.H
	Serum	C.H	C.H	C.H	M.H	M.H	M.H	M.H	M.H	M.H	M.H
6	Plasma	C.H	C.H	C.H	M.H	M.H	M.H	M.H	M.H	M.H	M.H
	Serum	C.H	C.H	M.H	M.H	M.H	M.H	M.H	M.H	M.H	M.H
7	Plasma	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H
	Serum	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H
8	Plasma	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H
	Serum	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H
9	Plasma	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H
	Serum	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H

10	Plasma	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H
	Serum	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H
11	Plasma	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H
	Serum	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H
12	Plasma	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H
	Serum	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H
13*	Plasma	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	V.M.H
	Serum	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	V.M.H	V.M.H
14*	Plasma	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H
	Serum	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	V.M.H	V.M.H
15*	Plasma	C.H	C.H	C.H	C.H	V.M.H	M.H	M.H	M.H	M.H	M.H
	Serum	C.H	V.M.H	M.H	M.H	M.H	M.H	M.H	M.H	M.H	M.H
16*	Plasma	C.H	C.H	C.H	C.H	V.M.H	M.H	M.H	M.H	M.H	M.H
	Serum	C.H	C.H	C.H	C.H	V.M.H	M.H	M.H	M.H	M.H	M.H
17*	Plasma	C.H	C.H	C.H	C.H	C.H	C.H	C.H	V.M.H	V.M.H	M.H
	Serum	C.H	C.H	C.H	C.H	C.H	C.H	C.H	V.M.H	V.M.H	M.H
18*	Plasma	C.H	C.H	C.H	V.M.H	M.H	M.H	M.H	M.H	M.H	M.H
	Serum	C.H	C.H	C.H	C.H	M.H	M.H	M.H	M.H	M.H	M.H

* 5 per cent suspension of sheep cells employed.

CONCERNING NATURAL THERMOSTABILE ANTISHEEP HEMOLYSIN IN HUMAN PLASMA AND SERUM

Technic. Plasmas and sera were heated in a water bath at 56°C. for one-half hour to remove complement and thermolabile antisheep hemolysin. During the process of heating, the plasmas usually became turbid and after standing a few hours showed numerous very small white masses which settled to the bottom of the test tubes and which appeared capable of absorbing small amounts of the antisheep hemolysin. After brief centrifuguing the clear plasmas were employed.

In conduction these tests plasma and serum were employed in varying doses of 0.05, 0.1 and 0.2 cc. with 1 cc. of 1:20 dilution of guinea-pig serum complement, 1 cc. of 2.5 per cent suspension of sheep cells followed by incubation in a water bath for one hour and readings the following day for the degree of hemolysis as an index to the presence of natural thermostable hemolysin.

Results. The results of these tests, given in table 10, show that four of the plasmas contained less natural hemolysin than the corresponding sera but more in two and an equal amount in twelve.

TABLE 10

Titration of normal thermostabile antisheep hemolysin in human plasmas and sera

NUMBER	SUBSTANCE	0.05 cc.	0.1 cc.	0.2 cc.
1	Plasma	N.H	N.H	N.H
	Serum	N.H	N.H	N.H
2	Plasma	N.H	V.S.H	S.H
	Serum	N.H	M.H	C.H
3	Plasma	S.H	M.H	C.H
	Serum	S.H	M.H	C.H
4	Plasma	S.H	M.H	C.H
	Serum	S.H	C.H	C.H
5	Plasma	C.H	C.H	C.H
	Serum	M.H	C.H	C.H
6	Plasma	N.H	N.H	N.H
	Serum	N.H	N.H	N.H

TABLE 10—*Continued*

NUMBER	SUBSTANCE	0.05 cc.	0.1 cc.	0.2 cc.
7	Plasma	C.H	C.H	C.H
	Serum	C.H	C.H	C.H
8	Plasma	S.H	M.H	M.H
	Serum	S.H	M.H	C.H
9	Plasma	C.H	C.H	C.H
	Serum	C.H	C.H	C.H
10	Plasma	C.H	C.H	C.H
	Serum	C.H	C.H	C.H
11	Plasma	C.H	C.H	C.H
	Serum	C.H	C.H	C.H
12	Plasma	N.H	N.H	N.H
	Serum	N.H	N.H	N.H
13	Plasma	S.H	S.H	S.H
	Serum	S.H	S.H	S.H
14	Plasma	S.H	M.H	M.H
	Serum	S.H	M.H	C.H
15	Plasma	C.H	C.H	C.H
	Serum	C.H	C.H	C.H
16	Plasma	C.H	C.H	C.H
	Serum	C.H	C.H	C.H
17	Plasma	N.H	N.H	N.H
	Serum	N.H	N.H	N.H
18	Plasma	S.H	S.H	S.H
	Serum	S.H	S.H	M.H

CONCERNING THE PRESENCE OF SYPHILIS ANTIBODY IN HUMAN PLASMA

Technic. These tests were conducted with inactivated plasmas and sera by a quantitative method (23) employing graded amounts of plasma and serum and as antigens an extract of

acetone insoluble lipoids and an alcoholic extract of ox heart re-enforced with cholesterin.

Results. The results of these tests are shown in table 11.

Of the plasmas and sera of eighteen persons tested, the plasmas and sera of six persons yielded negative Wassermann reactions; the remaining twelve yielded positive reactions of similar degree with both plasmas and sera. In several instances the plasmas yielded somewhat stronger reactions than the corresponding sera and notably in case 11.

TABLE 11

Quantitative Wassermann reactions with human plasmas and sera

NUMBER	SUBSTANCE	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.08 cc.	0.1 cc.	0.2 cc.	CONTROL 0.2 cc.
1	Plasma	−*	−	−	−		−	−	−
	Serum	−	−	−	−	−	−	−	−
2	Plasma	−	−	−	−	−	−	−	−
	Serum	−	−	−	−	−	−	−	−
3	Plasma	−	−	+4	+4	+4	+4	+4	−
	Serum	−	−	+3	+4	+4	+4	+4	−
4	Plasma	−	−	±	+1	+2	+3	+3	−
	Serum	−	−	±	±	+1	+2	+3	−
5	Plasma	−	−	−	−	−	−	−	−
	Serum	−	−	−	−	−	−	−	−
6	Plasma	−	−	+1	+2	+3	+3	+4	−
	Serum	−	−	+1	+2	+3	+3	+4	−
7	Plasma	−	−	±	+1	+2	+3	+3	−
	Serum	−	±	±	+1	+3	+3	+3	−
8	Plasma	−	−	−	−	−	−	−	−
	Serum	−	−	−	−	−	−	−	−
9	Plasma	+1	+4	+4	+4	+4	+4	+4	−
	Serum	+1	+3	+4	+4	+4	+4	+4	−
10	Plasma	±	+1	+4	+4	+4	+4	+4	−
	Serum	±	+1	+2	+3	+3	+3	+3	−

TABLE 11—*Continued*

NUMBER	SUBSTANCE	0.01 cc.	0.02 cc.	0.04 cc.	0.06 cc.	0.08 cc.	0.1 cc.	0.2 cc.	CONTROL 0.2 cc.
11	Plasma	−	±	$+^{1}$	$+^{1}$	$+^{2}$	$+^{2}$	$+^{3}$	−
	Serum	−	−	−	−	$+^{1}$	$+^{1}$	$+^{1}$	−
12	Plasma	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	−
	Serum	$+^{3}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	−
13	Plasma	±	$+^{1}$	$+^{3}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	−
	Serum	±	$+^{1}$	$+^{2}$	$+^{3}$	$+^{3}$	$+^{3}$	$+^{3}$	−
14	Plasma	±	$+^{3}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	−
	Serum	±	$+^{3}$	$+^{3}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	−
15	Plasma	−	$+^{2}$	$+^{3}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	−
	Serum	−	$+^{2}$	$+^{2}$	$+^{3}$	$+^{3}$	$+^{3}$	$+^{4}$	−
16	Plasma	−	$+^{1}$	$+^{1}$	$+^{2}$	$+^{3}$	$+^{4}$	$+^{4}$	−
	Serum	±	$+^{1}$	$+^{1}$	$+^{2}$	$+^{3}$	$+^{3}$	$+^{3}$	−
17	Plasma	−	−	−	−	−	−	−	−
	Serum	−	−	−	−	−	−	−	−
18	Plasma	−	−	−	−	−	−	−	−
	Serum	−	−	−	−	−	−	−	−

* − = complete hemolysis; ± = almost 10 per cent inhibition of hemolysis; + = 25 per cent inhibition of hemolysis; ++ = 50 per cent inhibition of hemolysis; +++ = 75 per cent inhibition of hemolysis; ++++ = 100 per cent inhibition of hemolysis.

CONCERNING THE PRESENCE OF NATURAL TYPHOID AGGLUTININ IN HUMAN PLASMAS AND SERA

Technic. A macroscopic technic was employed with inactivated plasmas and sera in amounts of 0.5 cc. of forty-eight hour broth cultures of a strain of *B. typhosus* followed by incubation at 55°C. for two hours and readings on the following day. The final dilutions of plasmas and sera were 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64; the usual culture controls were included in all experiments.

Results. The results are shown in table 12; minor differences were found in the quantity of natural typhoid agglutinin in plasmas and corresponding sera, but the results were closely similar and when different, the plasma usually contained a trace more of this antibody.

TABLE 12

Macroscopic agglutination tests with B. typhosus and human plasmas and sera for natural agglutinin

NUMBER	SUBSTANCE	1:2	1:4	1:8	1:16	1:32	1:64	ANTIGEN CONTROL
1	Plasma	+	+	−	−	−	−	−
	Serum	+	+	−	−	−	−	
2	Plasma	−	−	−	−	−	−	−
	Serum	−	−	−	−	−	−	
3	Plasma	+	±	−	−	−	−	−
	Serum	+	±	−	−	−	−	
4	Plasma	+	+	−	−	−	−	−
	Serum	+	+	−	−	−	−	
5	Plasma	+	+	−	−	−	−	−
	Serum	+	+	0	−	−	−	
6	Plasma	+	+	+	+	+	−	−
	Serum	+	+	+	+	+	−	
7	Plasma	+	+	−	−	−	−	−
	Serum	+	+	−	−	−	−	
8	Plasma	+	+	−	−	−	−	−
	Serum	+	+	+	−	−	−	
9	Plasma	+	+	+	−	−	−	−
	Serum	+	+	+	−	−	−	
10	Plasma	+	+	+	−	−	−	−
	Serum	+	+	+	−	−	−	
11	Plasma	±	−	−	−	−	−	−
	Serum	±	−	−	−	−	−	
12	Plasma	±	−	−	−	−	−	−
	Serum	−	−	−	−	−	−	
13	Plasma	+	+	+	+	+	+	−
	Serum	+	+	+	+	+	+	
14	Plasma	+	+	+	−	−	−	−
	Serum	+	+	+	−	−	−	

TABLE 12—*Continued*

NUMBER	SUBSTANCE	1:2	1:4	1:8	1:16	1:32	1:64	ANTIGEN CONTROL
15	Plasma	+	−	−	−	−	−	−
	Serum	−	−	−	−	−	−	
16	Plasma	+	+	+	+	−	−	−
	Serum	+	+	+	−	−	−	
17	Plasma	±	−	−	−	−	−	−
	Serum	−	−	−	−	−	−	
18	Plasma	+	+	+	+	±	−	−
	Serum	+	+	+	+	+	−	

CONCERNING THE PRESENCE OF COMPLEMENT AND NATURAL ANTIBODIES IN THE PLASMAS AND SERA OF NORMAL RABBITS

Studies exactly similar to those described were conducted with the plasmas and sera of normal rabbits, blood being collected from the carotid artery. The results of one experiment shown in table 13 indicate that rabbit plasmas contain as much or possibly more hemolytic complement than the corresponding sera; of the natural antibodies studied, namely, antisheep hemolysin, sheep hemagglutinin and typhoid agglutinin, plasma appeared to contain as much as serum. Of special interest in this connection is the observation that the heated plasma of a normal rabbit will yield a non-specific complement fixation reaction with Wassermann and bacterial antigens, to the same degree as the corresponding sera.

CONCERNING THE PRESENCE OF IMMUNE HEMOLYSIN AND HEMAGGLUTININ IN RABBIT PLASMAS AND SERA

Rabbits were immunized with repeated intravenous injections of washed sheep cells, bled from the carotid artery and the heated plasma and sera were titrated for antisheep hemolysin and hemagglutinin with 1 cc. of a 2.5 per cent suspension of washed sheep cells and water bath incubation for one hour. In the hemolysin titrations complement was furnished by guinea-pig serum in a dose of 1 cc. of a 1:20 dilution.

TABLE 13

*Titration of complement and natural antibodies on the plasma and serum of the normal rabbit**

1. Titration of complement

	1:10								
	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.5 cc.	0.6 cc.	1.0 cc.	1.5 cc.	2.0 cc.
Plasma	N.H	N.H	N.H	N.H	V.S.H	V.S.H	C.H	C.H	C.H
Serum	N.H	N.H	N.H	N.H	V.S.H	V.S.H	M.H	C.H	C.H

2. Titration of natural thermostabile antisheep hemolysin

	5 PER CENT SHEEP CELLS									
	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.5 cc.	0.6 cc.	0.7 cc.	0.8 cc.	0.9 cc.	1.0 cc.
Plasma	C.H	C.H	C.H	M.H	M.H	M.H	M.H	M.H	M.H	M.H
Serum	C.H	C.H	C.H	M.H	M.H	M.H	M.H	M.H	M.H	M.H

0.1 cc. of plasma and serum employed.

3. Titration of hemagglutinin for sheep cells

	FINAL DILUTIONS						
	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Plasma	+	+	−	−	−	−	−
Serum	+	−	−	−	−	−	−

4. Agglutination tests with B. typhosus

	FINAL DILUTIONS						
	1:2	1:4	1:6	1:8	1:12	1:24	1:32
Plasma	−	−	−	−	−	−	−
Serum	−	−	−	−	−	−	−

5. Non-specific typhoid complement fixation

	1:10							Control 2.0 cc.
	0.1 cc.	0.2 cc.	0.4 cc.	0.6 cc.	0.8 cc.	1.0 cc.	2.0 cc.	
Plasma	−	$+^1$	$+^2$	$+^3$	$+^3$	$+^4$	$+^4$	M.H
Serum	−	$+^1$	$+^2$	$+^3$	$+^3$	$+^4$	$+^4$	M.H

6. Non-specific Wassermann reaction

	1:10							Control 2.0 cc.
	0.1 cc.	0.2 cc.	0.4 cc.	0.6 cc.	0.8 cc.	1.0 cc.	2.0 cc.	
Plasma	−	−	$+^1$	$+^2$	$+^3$	$+^4$	$+^4$	M.H
Serum	−	−	$+^1$	$+^2$	$+^3$	$+^4$	$+^4$	M.H

* The respective tests were conducted after the same technic previously described.

The results are indicated in table 14 and they have shown that the plasma usually contained appreciably more hemolysin and hemagglutinin than the corresponding sera.

TABLE 14

Titration of immune antisheep hemolysin in rabbit plasmas and sera

NUMBER		DILUTED 1:5000									
		0.05 cc.	0.1 cc.	0.15 cc.	0.2 cc.	0.25 cc.	0.3 cc.	0.35 cc.	0.4 cc.	0.45 cc.	0.5 cc.
14	Plasma	S.H	M.H	V.M.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H
	Serum	S.H	M.H	M.H	V.M.H	V.M.H	C.H	C.H	C.H	C.H	C.H
80	Plasma	M.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	V.H
	Serum	S.H	V.M.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H

TABLE 15

Titration of sheep hemagglutinin in the plasmas and sera of immunized rabbits

NUMBER	SUBSTANCE	FINAL DILUTIONS						
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280
14	Plasma	+	+	+	+	+	−	−
	Serum	+	+	+	−	−	−	−
80	Plasma	+	+	+	−	−	−	−
	Serum	+	+	−	−	−	−	−

CONCERNING THE PRESENCE OF IMMUNE TYPHOID AGGLUTININ AND COMPLEMENT FIXING ANTIBODY IN RABBIT PLASMAS AND SERA

Rabbits were immunized with repeated intravenous injections of heated typhoid vaccine and bled from the carotid artery, the plasmas and sera being titrated for immune agglutinin and complement fixing antibody with heated and unheated plasmas and sera and with the usual controls.

The results shown in tables 16 and 17 demonstrate that the plasmas always contained an equal or sometimes greater amount of agglutinin and always an equal amount of complement fixing antibody.

TABLE 16

Macroscopic agglutination tests of the plasmas and sera of the immunized rabbits against B. typhosus

NUMBER	SUBSTANCE	FINAL DILUTIONS											
		1:1000	1:1600	1:2000	1:3200	1:4000	1:5000	1:6000	1:7000	1:7200	1:8000	1:9000	1:10000
84	Plasma	+	+	+	+	−	−	−					
	Serum	+	+	−	−	−	−	−					
2	Plasma	+	+	+	+	+	+	+	+	+	+	+	−
	Serum	+	+	+	+	+	+	+	+	+	+	±	−
3	Plasma	+	+	+	+	+	+	±	±	±	−	−	−
	Serum	+	+	+	+	+	+	±	±	−	−	−	−

TABLE 17

Thyroid complement fixation tests with the plasmas and sera of immunized rabbits

NUMBER	SUBSTANCE		DOSES OF UNDILUTED PLASMA AND SERUM												
			0.0002 cc.	0.0004 cc.	0.0005 cc.	0.0008 cc.	0.001 cc.	0.002 cc.	0.004 cc.	0.008 cc.	0.01 cc.	0.04 cc.	0.1 cc.	0.2 cc.	Control 0.2 cc.
84	Heated	Plasma			−	−	−	±	+[2]	+[4]	+[4]	+[4]	+[4]	+[4]	−
		Serum			−	−	−	±	+[2]	+[4]	+[4]	+[4]	+[4]	+[4]	−
	Unheated	Plasma			±	±	+[1]	+[2]	+[3]	+[4]	+[4]	+[4]	+[4]	+[4]	−
		Serum			±	±	+[1]	+[2]	+[4]	+[4]	+[4]	+[4]	+[4]	+[4]	−
															0.01
2	Heated	Plasma	−	−		+[1]	+[2]		+[4]	+[4]	+[4]				−
		Serum	−	−		+[1]	+[2]		+[4]	+[4]	+[4]				−
	Unheated	Plasma	−	±		+[1]	+[3]		+[4]	+[4]	+[4]				−
		Serum	−	±		+[1]	+[3]		+[4]	+[4]	+[4]				−
3	Heated	Plasma	−	−		±	+[2]		+[4]	+[4]	+[4]				−
		Serum	−	−		±	+[2]		+[4]	+[4]	+[4]				−
	Unheated	Plasma	−	−		±	+[2]		+[4]	+[4]	+[4]				−
		Serum	−	−		±	+[2]		+[4]	+[4]	+[4]				−

SUMMARY

A brief summary of the results of this study presented in tables 1 to 17 is given in table 18 which shows in parallel columns the comparative results of tests for hemolytic complement, various natural and immune antibodies in human and rabbit plasmas and sera. In the majority of instances the various plasmas contained an equal or greater amount of hemolytic complement or antibodies than were present in the corresponding sera, and only exceptionally lesser amounts.

TABLE 18

Summary of tables showing comparative results with plasmas and sera

SOURCE	SUBSTANCE	COMPARATIVE RESULTS	
		Plasma	Serum
18 Human	Hemolytic complement	Average unit 0.0312 cc.	Average unit 0.0412 cc.
18 Human	Total hemolytic activity for sheep cells	Equal or greater in 90 per cent	Weaker in 10 per cent
18 Human	Thermostabile antisheep hemolysin	Equal in about 78 per cent	Greater in 22 per cent
18 Human	Syphilis antibody	Positive in 12	Positive in 12
18 Human	Natural typhoid agglutinin	Equal	Equal
Rabbit	Hemolytic complement	Unit 0.1 cc.	Unit 0.15 cc.
Rabbit	Total hemolytic activity for sheep cells	0.3 cc. cells	0.3 cc. cells
Rabbit	Sheep hemagglutinin	1:4	1:2
Rabbit	Natural typhoid agglutinin	None	None
Rabbit	Non specific typhoid complement fixation	(+) 0.02 cc.	(+) 0.02 cc.
Rabbit	Non specific Wassermann reaction	(+) 0.04 cc.	(+) 0.04 cc.
Rabbit	Immune antisheep hemolysin	1:25000	1:16000
Rabbit	Immune antisheep hemolysin	1:50000	1:33000
Rabbit	Immune sheep hemagglutinin	1:320	1:80
Rabbit	Immune sheep hemagglutinin	1:80	1:40
Rabbit	Immune typhoid agglutinin	1:3200	1:1600
Rabbit	Immune typhoid agglutinin	1:9000	1:9000
Rabbit	Immune typhoid agglutinin	1:7200	1:7000
Rabbit	Specific typhoid complement fixation	(+) 0.002 cc.	(+) 0.002 cc.
Rabbit	Specific typhoid complement fixation	(+) 0.0008 cc.	(+) 0.0008 cc.
Rabbit	Specific typhoid complement fixation	(+) 0.0008 cc.	(+) 0.0008 cc.

CONCLUSIONS

1. A method for the collection of plasma is described which has yielded uniform success and simplified this difficult technical problem. This technic involves the use of dried sodium oxalate after a method devised by Meeker, and a paraffined tube.

2. Sodium oxalate in the proportion of 0.001 gram per cubic centimeter of blood prevents coagulation and does not exert any injurious influence upon hemolytic complement or antibodies; in amounts of 0.004 gram per cubic centimeter of blood sodium oxalate may prove to be anticomplementary.

3. The oxalated plasmas of normal and syphilitic persons and normal and immunized rabbits contains hemolytic complement in the same or somewhat greater amounts than the corresponding sera.

4. The oxalated plasmas of syphilitic persons contain the same amounts of Wassermann antibody as the corresponding sera.

5. The oxalated plasmas of persons and rabbits contain the same or occasionally slightly greater amounts of such natural antibodies as antisheep hemolysin and typhoid agglutinin as the corresponding sera.

6. The oxalated plasmas of normal and immunized rabbits contained as much specific and non-specific complement fixing substances and specific bacterial and hemagglutinins as the corresponding sera.

7. The general conclusion of this investigation is that hemolytic complement and natural and immune antibodies exist free and preformed in the circulating plasma of the blood.

I beg to express my appreciation to Prof. Kolmer for directions and aid in conducting this work.

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A METHOD FOR THE PRODUCTION OF A HOMOGENEOUS SUSPENSION OF BACILLUS ANTHRACIS TO BE USED IN AGGLUTINATION REACTIONS

ARLYLE NOBLE

From the Research Laboratory of Parke, Davis and Company, Detroit, Michigan

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Because of the fact that an accurate and reliable method for the standardization of antianthrax serum has not yet been developed, while the necessity for such a method is very apparent, experiments were undertaken to make use of the agglutination and complement fixation reactions. So far the results obtained from the animal protection tests have not proved uniform, and therefore not satisfactory, mainly because the animals on which the serum is being tested are so highly susceptible to the disease, and because of the difficulty in standardizing the test culture and maintaining a standard dose, due to variations in the virulence of different strains of *B. anthracis*.

Of the serum reactions, the complement fixation test has thus far not proven to be of value, as a stable antigen has not been produced, though further experiments are being made along this line. With regard to agglutination, the results as reported in the literature appear quite conflicting. Sobernheim in reviewing the subject states that the agglutinating action of serum on anthrax bacilli may be observed both microscopically and macroscopically, though the immobility of the bacilli and their inclination to arrange themselves in clumps make the judgment of agglutination difficult; and that with many sera one always obtains distinct agglutination in strong dilution, while, on the other hand, it is often lacking in even high grade anthrax serum. He gives the statements of various writers, briefly, as follows: Sawtschenko found that horse serum agglutinated in every case,

irrespective of whether it came from normal or preventatively inoculated animals, while dog serum of both catagories never evidenced agglutinating power. Contrary to the report made by Sobernheim, that the specifically agglutinating power is usually lacking in the anthrax serum, Cavine states that a series of various anthrax sera proved active, in dilutions of 1–50,000 to 150,000 even 1–500,000. Gottstein obtained completely negative results in a retest of these experiments, several high grade sera from horses, cattle and sheep showing no agglutination power. Sobernheim states that the remarkable fact can be determined that a serum agglutinates, for example, the bacilli of virulent anthrax and of Pasteur's Vaccine I, but not of Vaccine II, while another perhaps has no influence on the virulent anthrax and Vaccine II, but gives a distinct agglutination with cultures of Vaccine I.

From these conflicting statements Sobernheim draws this conclusion:

> Although the question of agglutination for anthrax serum requires further explanation, we may say, that a parallelism exists between the agglutinating and immunizing power of the serum in no case, and the presence or absence of agglutinating action has absolutely no connection with the degree of immunity in the serum producing animal.

It seems to the writer that the problem is largely, if not entirely, a problem of the suspension. Because of the nature of the organism, growing as it does in long chains and producing spores, it does not easily lend itself to agglutination experiments. However, a homogenous suspension of *B. anthracis* has been prepared and an increase of agglutinins demonstrated in sera from horses treated with vaccines of *B. anthracis*, as against sera from normal horses.

The suspension for the agglutination tests was prepared as follows: The cultures employed were four strains of *B. anthracis* furnished by the United States Bureau of Animal Industry, and designated by them "Davis," "Chestertown," "N. H.," and "6071." These four strains were transplanted

daily for ten days on plain agar and incubated at 42.5°C., until a sporeless and very vigorous growth was obtained. Each strain was then planted on plain agar in quart whiskey flasks and incubated for twelve hours at 42.5°C. The growths were washed off in physiologic salt solution containing 0.5 per cent formalin (about 100 cc. to a flask). The suspensions were shaken in a mechanical shaker for forty-eight hours. After standing for several days and being tested for sterility, equal parts of each suspension were mixed in a cylinder; shaken for twenty-four hours; and allowed to stand over night. The larger clumps settle out, leaving a homogeneous suspension above. This upper portion was poured off and filtered several times through four thicknesses of sterile cheesecloth. The suspension was then diluted with physiologic salt solution plus 0.5 per cent formalin to a density corresponding to a suspension of *B. typhosus* containing 2000 million bacteria per cubic centimeter. A suspension of *B. anthracis* so prepared is perfectly homogeneous, stands up for at least forty-eight hours at 37°C. and shows no spontaneous agglutination.

The sera used were from thirteen horses which were treated first with vaccines of attenuated cultures and then with increasing doses of virulent *B. anthracis*. The strains were the same as used in the preparation of the suspensions. Also, sera from seven normal, untreated horses were tested.

The agglutination tests. All the agglutination tests were macroscopic. In carrying out the tests, the serum dilutions were made in test-tubes with physiologic salt solution. The dilutions were never started with less than 1 cc. of undiluted serum and the volume of each dilution was always more than sufficient for the test. Special pipets, graduated to 0.5 and 1 cc. were employed throughout and a different pipet was used for each dilution. All glass-ware used in connection with the tests was clean and sterile. In the test, each small agglutination tube contained 0.5 cc. of suspension plus 0.5 cc. of diluted serum, with a control tube containing 0.5 cc. suspension plus 0.5 cc. salt solution. The tests were incubated at 37°C. for twenty-four hours.

TABLE 1

SERUM DILUTION	RESULTS, JANUARY 17, 1919						
	Suspension — B. anthracis Sera —						
	Normal horse 901	Antianthrax, Horse					
		1057	1051	1050	1049	1047	1045
1–10	+++	+++	+++	+++	+++	+++	+++
1–20	+++	+++	+++	+++	+++	+++	+++
1–40	+++	+++	+++	+++	+++	+++	+++
1–80	++	+++	+++	+++	+++	+++	+++
1–200	+	+++	+++	+++	+++	+++	+++
1–400	−	+++	+++	+++	+++	+++	+++
1–800	−	+++	+++	+++	+++	+++	+++
1–1600	−	+++	+++	+++	+++	+++	+++
1–2000	−	+++	+++	+++	+++	+++	+++
1–3200		+++	+++	+++	++	++	+++
1–6400		+++	++	++	+	++	+
1–10000		++	+	−	+	+	+
1–20000		+	−	−	−	+	−
1–40000		−	−	−	−	−	−
Control	−	−	−	−	−	−	−

+++ represents complete agglutination; ++ partial; + slight agglutination, but still with positive clumping.

The results of the agglutination reactions with antianthrax sera and normal horse sera are given in tables 1 and 2.

Each antiserum has been tested more than once and different bleedings fom the same horse have been tested with practically no variation in the agglutination titer.

The sera from five normal horses, in addition to the two given in the tables, gave agglutination titers of from 1 in 80 to 1 in 200.

SUMMARY

A satisfactory suspension of *B. anthracis*, for agglutination reactions, has been prepared by the described method. In order to be assured of a homogeneous suspension certain points must be observed. The cultures must be sporeless and must contain vigorous growths free from old organisms. The growths

TABLE 2

SERUM DILUTION	RESULTS, JANUARY 18, 1919							
	Suspension — B. anthracis Sera —							
	Normal horse 1665	Antianthrax, Horse						
		1042	1027	1025	957	956	955	953
1–10	+++	+++	+++	+++	+++	+++	+++	+++
1–20	+++	+++	+++	+++	+++	+++	+++	+++
1–40	++	+++	+++	+++	+++	+++	+++	+++
1–80	+	+++	+++	+++	+++	+++	+++	+++
1–200	−	+++	+++	+++	+++	+++	+++	+++
1–400	−	+++	+++	+++	+++	+++	+++	+++
1–800	−	+++	+++	+++	+++	+++	+++	+++
1–1600	−	+++	+++	+++	+++	+++	+++	+++
1–2000	−	+++	+++	+++	+++	+++	+++	+++
1–3200		+++	+++	++	+++	+++	+++	+++
1–6400		++	++	++	++	+	++	+++
1–10000		+	+	+	+	−	++	++
1–20000		−	−	−	−	−	+	+
1–40000		−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−

for the suspensions must be young, not more than eighteen hours old. The suspension must be thoroughly shaken; the larger clumps allowed to settle; and then carefully strained.

Agglutinins have been demonstrated in the serum from horses hyper-immunized with *B. anthracis*. The antianthrax sera from thirteen horses have given agglutination titers of from 1 in 6400 to 1 in 20,000, as against titers of from 1 in 80 to 1 in 200 in normal horses.

The agglutination tests show that certain antibodies have been produced in horses treated with *B. anthracis* and, in the absence of a satisfactory animal protection test or method of complement fixation the agglutination test may be used as a method for standardizing antianthrax serum.

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ON THE NATURE OF ECLAMPSIA

ISEI OBATA

From the Forensic Institute of the Imperial University of Tokio

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As is well known Dold (1) discovered, in the salt-solution extract of viscera, a poisonous property, which can be neutralized by blood serum. This so-called "Organgift" still remains a problem attracting the attention of observers. As to the nature and properties of the poison we have a number of other investigations (Dold and Ogata (2), Ascoli (3), Isar and Patane (4), Ichikawa (5), Aronson (6), Dold and Kodama (7), Yoshimura,[1] Goto[1] and Ishikawa),[1] the results of which diverge from one another.

It would seem probable that the toxin above mentioned is contained also in the placenta, which may be considered as one of the viscera. The placenta is, in fact, a viscus which is present in the female sex only during a certain period of life and which is often placed anatomically in the category of tumor; it extends its villi into the maternal blood in which they are bathed so long as they exist, thus standing in a relation to the maternal body that is quite peculiar to itself. This relationship suggests the possibility of a resulting peculiar pathological process; it also provides an excellent material for study because of the ultimate natural separation of the organ from its site. It seemed possible, in view of the peculiar relationship referred to, that if the placenta does contain the poisonous property or substance of Dold, these could, under some unusual circumstances, be responsible for the pathology and the symptom-complex of eclampsia.

[1] In Japanese only.

The present study was pursued from this point of view.

The question whether extracts of the placenta are poisonous was first investigated.

PREPARATION OF THE PLACENTAL EXTRACT

As soon as the placenta was expelled the umbilical cord was cut off together with that portion of the placenta which surrounds its attachment to it. From the so prepared placenta as much blood as possible was expressed and the decidual tissue was also removed. The cotyledon, preferably that portion most deficient in large villi was taken by weight, cut into pieces and ground in a mortar, being mixed, at last, with 0.85 per cent salt solution in a proportion of 1 to 3 parts by weight. The mixture, after being stirred, was left half an hour at room temperature before being filtered through the *habutai*, a fine silk. The filtrate was centrifuged and the supernatant fluid, which was designated placental extract, was used for our experiment. The extract was opalescent and of a pale pink color and it contained no solid particles in suspension. The placental extract employed in the present investigation was usually prepared from fresh placentae taken immediately after birth; but in some instances extracts were prepared from placentae which had been left in a refrigerator for a period that never exceeded seven hours.

TOXIC SYMPTOMS IN THE EXPERIMENTAL ANIMALS

The animals used in the present experiment were generally the Japanese dancing mice (a variety of *Mus musculus*), although rabbits were often employed. The following description applies to the mice, unless otherwise stated. The injections were made into the caudal veins.

After the injection of a lethal dose of the extract there was an interval of ten to thirty seconds, rarely a minute before the animal became excited and fell at once in a brief clonic or, rarely, tonic convulsion, which was succeeded by a violent dyspnoea, coma and finally death within one to three minutes

after the beginning of the convulsion. While the symptoms just described occurred in the majority of instances there were occasional exceptions in which the effects were more prolonged, death following sometimes after hours or days. Even in these cases, however, two symptoms were almost always noticeable; namely, dyspnoea and convulsions.

COMPARISON, AS TO TOXICITY, OF THE NORMAL PLACENTA WITH THAT OF THE ECLAMPTIC INDIVIDUAL

As the resistance exhibited by individual mice to the placental "poison" varied widely, it was necessary to make duplicate injections of each dose of the extract, in order to control this varying resistance. The lethal dose was taken as the minimal dose which killed both the individuals; in case a certain dose was able to kill only one of the two, the lethal dose is taken as an average of this dose and of that which was able to kill both animals.

In table 1 are listed the cases in which the experimental animals died within ten hours after the injection; those animals which either recovered from the dyspnoea, convulsion and other symptoms caused by the injection, or died after surviving more than 10 hours are designated with the term "reaction." The animals designated "dead" were those which died in most cases within several minutes, though some survived two or three hours and a few lived still longer, but not beyond ten hours. As shown by repeated experiments, no constant relation has been observed between the dose injected and the body weight of the mice employed in the experiments, which varied from 7 grams to 15 grams; hence the body weight is omitted in the descriptions as well as in the tables.

The results presented in table 1 show that the lethal dose of the extract from normal placentae varies from 0.025 to 0.15 cc. and that from eclamptic placentae varies from 0.019 to 0.1 cc. It follows that the extracts of normal placentae can hardly be distinguished from those of eclamptic placentae with respect to toxic property.

TABLE 1

	NAMES OF PERSONS WHOSE PLACENTAE WERE USED IN THE EXPERIMENTS	DOSE OF PLACENTAL EXTRACT INJECTED						AVERAGE LETHAL DOSE
		0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.0125 cc.	0.0062 cc.	
								cc.
Placenta of normal women.....	S. Ts.		dead	dead	dead	—		0.025
				dead	dead	—		
	T. I.		dead	dead	—			0.05
				dead	—			
	M. N.		dead	dead	dead	dead	—	0.0313
			dead	—	dead	reaction	—	
	N. A.	dead	dead	—				0.15
		dead	reaction	reaction				
	M. H.	dead	dead	dead	reaction		—	0.125
		dead	reaction	reaction	reaction			
	K. O.	dead	reaction	—				0.15
		dead	dead	reaction				
	K. S.		dead	dead	—	—		0.0625
			dead	reaction	dead	—		
	T. K.	dead	reaction	dead	—			0.125
		dead	reaction	reaction	—			
	I. M.	dead	reaction	reaction	—			0.125
		dead	dead	dead	—			
	S. O.		dead	reaction	—			0.1
			dead	reaction	—			
	M. Īg.		dead	dead	—			0.05
			dead	dead	—			
	T. Ya.		dead	reaction	—	—		0.0625
			dead	dead	dead	—		
	A. Y.		dead	reaction	—			0.1
			dead	reaction	—			
	M. K.	dead	dead	reaction	reaction	—		0.0625
			dead	reaction	dead	—		

Placenta of eclamptic women	S. T.		dead	dead	dead	—	reaction	0.019
			dead	dead	dead	dead	—	
	T. M.		dead	dead	reaction	—		0.05
			dead	dead	—	—		
	M. I.		dead	dead	dead	—		0.025
			dead	dead	dead	—		
	H. Y.			dead	reaction	dead	—	0.032
				dead	dead	—	—	
	S. Y.			dead	—	—	—	0.031
				dead	dead	dead	—	
	T. Y.	dead	dead	reaction	—	—		0.1
		dead	dead	reaction	reaction	—		
	R. Y.		dead	dead	—	—		0.0375
			dead	dead	dead	—		

Note. "dead" means death of experimented animals within twelve hours after injection; "dead n. d." signifies the death with forty-eight hours; by "reaction" is meant survival beyond forty-eight hours; while the cases without reaction are marked a dash ("—".)

TABLE 2

	NAMES OF PERSONS WHOSE SERA WERE EMPLOYED IN THE EXPERIMENTS	PERIOD AT WHICH SERUM HAD BEEN TAKEN	DOSE OF SERUM INJECTED					AVERAGE LETHAL DOSE
			0.5 cc.	0.4 cc.	0.3 cc.	0.2 cc.	0.1 cc.	
								cc.
Men	S. T.		dead	dead	—			0.4
	T. H.		dead		dead	—		0.45
					dead	—		
	K. A.			dead	dead	dead	—	0.25
				dead	dead	—	—	
	T. T.			dead	dead	dead	—	0.25
				dead	dead	—	—	
	O. A.		dead	dead	dead	—		0.45
Non-gravida	Y. M.				dead	reaction	—	0.25
				dead	dead	dead	—	
	T. E.			dead	reaction	dead	—	0.3
				dead	dead	reaction	—	
	C. H.				dead	dead	—	0.2
					dead	dead	—	
	M. O.				dead	dead	—	0.25
					dead	reaction	—	
	I. I.			dead	—	—	—	0.3
				dead	reaction	dead	—	
Healthy gravida	M. I.	6th month of pregnancy		dead	dead	—	—	0.3
					dead	reaction	—	
	O. O.	7th month of pregnancy	dead	dead	reaction	—		0.4
				dead	—	—		

Healthy gravida	K. N.	8th month of pregnancy			dead dead	dead —	— —	0.25
	S. K.	8th month of pregnancy	reaction	dead —	reaction dead			
	K. Ko.	10th month of pregnancy		dead dead	— reaction	— —	— —	0.4
	K. T.	10th month of pregnancy			dead dead	— —	—	0.3
Healthy puerperal women	H. N.	$1\frac{1}{2}$ hours after labor		dead	dead dead	— —		0.3
	N. N.	3rd hour after labor	dead dead	reaction reaction	reaction dead	— —		0.4
	K. K.	20th hour after labor	dead dead	— dead	dead —	— —		0.4
	T. Ma.	23rd hour after labor			dead dead	dead dead	— —	0.2
	K. M.	2nd day after labor		dead	dead	—	—	0.3
	K. S.	3rd day after labor			dead dead	dead —	— —	0.25
	R. N.	5th day after labor			dead dead	— dead	— —	0.25
	N. T.	5th day after labor				dead dead	— —	0.2
	S. S.	6th day after labor		dead		dead dead	— —	0.2
	N. Y.	6th day after labor			dead dead	— —		0.3
	K. O.	7th day after labor			dead dead	dead dead	— —	0.25
	M. Y.	8th day after labor		dead	dead	—		0.3
	M. K.	8th day after labor			dead dead	reaction —	— —	0.3

TABLE 2—*Continued*

	NAMES OF PERSONS WHOSE SERA WERE EMPLOYED IN THE EXPERIMENTS	PERIOD AT WHICH THE SERUM HAD BEEN TAKEN	DOSE OF SERUM INJECTED					AVERAGE LETHAL DOSE
			0.5 cc.	0.4 cc.	0.3 cc.	0.2 cc.	0.1 cc.	
Eclamptic Women.....	S. T.	Before labor		dead	dead	dead	—	0.3
		During attack		dead	reaction	reaction	—	
		11th day after labor		dead	dead	—	—	0.25
				dead	dead	dead	—	
	T. M.	Before labor			dead	dead	—	0.2
		During attack			dead	dead	—	
		3rd day after labor			dead	dead	—	0.2
					dead	dead	—	
	M. I.	Before labor			dead	dead	dead	0.15
		During attack			dead	dead	—	
		5th day after labor			dead	dead	dead	0.15
					dead	dead	—	
		9th day after labor			dead	dead	dead	0.2
					dead	—	—	
	H. Y.	Just after labor			dead	dead	—	0.2
		During attack			dead	dead	—	
		$3\frac{1}{2}$ days after labor			dead	dead	—	0.2
					dead	dead	—	

Eclamptic Women.....	S. Y.	Before labor			dead	—	—	0.3
		During attack			dead	—	—	
		4th day after labor			dead	—	—	0.25
					dead	dead	—	
	T. Y.	Just after labor			dead	dead	—	0.2
		During attack			dead	dead	—	
		9th day after labor			dead	dead	—	0.2
					dead	dead	—	
	R. Y.	Before labor			dead	dead	—	0.2
		During attack			dead	dead	—	
		$3\frac{1}{2}$ days after labor			dead	—	—	0.3
					dead	—	—	
		$6\frac{1}{2}$ days after labor			dead	dead	—	0.2
					dead	dead	—	

COMPARISON OF THE TOXICITY OF THE SERUM OF NORMAL AND ECLAMPTIC INDIVIDUALS

Fresh serum, when injected intravenously into the dancing mouse is toxic, causing symptoms which differ only slightly from those produced with placental extract. It was noticeable, however, that there was a longer interval between the injection and the onset of symptoms in the case of the serum than there was with the extract.

In table 2 are presented the results of the comparative tests of the toxicity of fresh serum from men and from normally pregnant or puerperal women, as well as from eclamptic women.

These results show that no significant difference exists in the toxicity of serum from individuals of the different groups. Furthermore, it is seen that no such difference exists between the serum obtained during the attack of eclampsia and that obtained after recovery from that condition.

COMPARISON OF THE SERA OF NORMAL AND ECLAMPTIC INDIVIDUALS WITH RESPECT TO THEIR CAPACITY TO NEUTRALIZE THE TOXIC PROPERTY OT THE PLACENTAL EXTRACT

The foregoing experiments had revealed no differences between the materials obtained from the eclamptic individuals and those derived from normal individuals. However, as the following experiment will show, such difference was found in the capacity of the serum to neutralize the poisonous property of the placental extract.

The tests of the neutralizing power of the sera were conducted by mixing 1 cc. of the placental extract with quantities of the fresh serum varying from 0.7 cc. to 0.025 cc., physiological salt solution being added to bring the total volume of the mixtures up to 2 cc. These mixtures were injected after an incubation of one hour at 37°C. The results of these tests are presented in table 3.

It is seen that normal human serum, whether from men or from women that are pregnant or not in that condition, possesses a practically uniform power of neutralizing the poisonous property of placental extract, 0.2 to 0.3 cc. of such serum sufficing to inhibit the action of 1 cc. of extract. On the other hand, *this neutralizing power is considerably less in the serum of eclamptic women during the attack,* the normal power being restored after the individual has recovered from the condition. As much as 0.6 cc. of the serum taken during the attack, was usually required to neutralize the toxic action of 1 cc. of placental extract, whereas after recovery 0.3 or 0.4 cc., sometimes as little as 0.2 cc. being sufficient. The normal neutralizing power of the blood was found to be restored by the fourth or fifth day of the puerperium in eclamptic women.

In interpreting the diminished neutralizing power of the serum of eclamptic individuals, which we have observed, one may consider this change as either a cause or an effect of the symptoms, and the possibility that the change referred to was a result of the convulsions was experimentally investigated. To this end the sera of three rabbits were examined as to their neutralizing power before and after a toxic injection of placental extract, which produced convulsions in the three animals. The results of this experiment, which are presented in table 4, show that, in the rabbit, convulsions do not alter the neutralizing power of the serum.

We have investigated the question whether a power of neutralizing placental poison is developed in the blood of human individuals by an immunological process. This question was studied by comparing the neutralizing power of the serum of pregnant and non-pregnant women. The results of that comparison, which are presented in table 5, show that the neutralizing power of the blood is not increased during pregnancy. Hence, no evidence was furnished, by the study, of an immunological origin of a neutralizing power of the blood.

TABLE 3

	NAMES OF PERSONS FROM WHOM SERUM HAD BEEN TAKEN FOR THE EXPERIMENTS	EXPERIMENTAL FLUID / SERUM / PHYSIOLOGICAL SALT SOLUTION / PLACENTAL EXTRACT	I 0.6 0.4 1.0	II 0.5 0.5 1.0	III 0.4 0.6 1.0	IV 0.3 0.7 1.0	V 0.2 0.8 1.0	VI 0.1 0.9 1.0	VII 0.05 0.95 1.0	VIII 0.025 0.975 1.0	CONTROL 1.0 1.0
Men	K. A.				—	—	—	dead	dead	reaction	dead
	T. H.				—	—	—	dead	dead	dead	reaction
	O. A.				—	—	—	reaction	dead	dead	dead
	H. H.				—	—	—	dead	reaction	reaction	dead n.d.
	M. K.				—	—	—	dead n.d.	reaction	dead	dead
				—	reaction	reaction	dead	dead	dead	dead n.d.	dead
				—	dead	dead	dead	dead	dead n.d.	reaction	dead
					—	—	dead	dead	dead n.d.	dead	dead
					—	—	dead n.d.	dead	dead	dead	dead
Non-gravida	C. H.				—	—	—	dead	dead	dead	dead
	A. A.				—	—	—	dead	dead	reaction	dead
	T. Y.				—	—	dead	dead	dead	dead	dead
	I. I.				—	—	dead n.d.	reaction	dead	dead	dead
	M. O.				—	—	—	dead n.d.	reaction	dead	dead n.d.
					—	—	reaction	dead n.d.	dead	dead	dead
						—	—	dead	reaction	dead	dead
						—	—	dead	dead	reaction	dead
						—	—	dead	dead	dead	dead
						—	—	dead	dead	dead	dead

Healthy gravida	M. S.	7th month of pregnancy			—	dead	dead	dead	dead n.d.	dead	dead
					—	reaction	dead	dead	dead	dead	dead
	T. S.	7th month of pregnancy				—	—	reaction	dead	dead	dead
						—	—	reaction	dead	reaction	dead
	K. N.	8th month of pregnancy				—	—	reaction	dead	dead	dead
						—	—	dead	reaction	dead	dead
	S. K.	8th month of pregnancy			—	—	dead	dead	reaction	dead	dead
					—	—	dead n.d.	dead	dead	dead	dead
	K. Ko.	10th month of pregnancy				—	—	reaction	dead	dead	dead
						—	—	dead	dead	dead	dead
Healthy puerperal women	H. N.	$1\frac{1}{2}$ hour. After labor				—	—	reaction	dead	dead	dead
						—	—	dead	dead	reaction	reaction
	T. H.	$1\frac{1}{2}$ hour after labor			—	—	dead	dead	dead	dead	dead
					—	—	dead n.d.	dead n.d.	dead	dead n.d.	reaction
	C. N.	3rd hour after labor			—	—	reaction	dead	dead	dead	dead
					—	—	reaction	dead	dead	dead	dead
	K. K.	20th hour after labor				—	—	reaction	reaction	dead	dead
						—	—	dead n.d.	dead	reaction	dead
	T. Ma.	23rd hour after labor			—	—	reaction	dead	dead	reaction	dead
					—	dead n.d.	dead	dead	dead	dead	dead
	R. N.	5th day after labor				—	—	—	dead	—	reaction
						—	—	reaction	dead	reaction	dead
	M. H.	5th day after labor				—	dead	dead	dead	dead	dead
						—	dead	dead	dead	dead	dead

TABLE 3—*Continued*

	NAMES OF PERSONS FROM WHOM SERUM HAD BEEN TAKEN FOR THE EXPERIMENTS	EXPERIMENTAL FLUID	I	II	III	IV	V	VI	VII	VIII	CONTROL
		SERUM	0.6	0.5	0.4	0.3	0.2	0.1	0.05	0.025	
		PHYSIOLOGICAL SALT SOLUTION	0.4	0.5	0.6	0.7	0.8	0.9	0.95	0.975	1.0
		PLACENTAL EXTRACT	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Eclamptic women	S. T.	Before labor	—	dead	dead	dead	dead	dead	dead	dead	dead
		During attack	—	dead	dead	dead	dead	dead	dead	dead	dead
		11th day after labor	—	—	—	—	dead	dead	dead	dead	dead
			—	—	—	—	dead	dead	dead	dead	dead
	T. M.	Before labor	—	dead	dead	dead	dead	dead n.d.	dead n.d.		dead
		During attack	—	dead	dead n.d.	dead n.d.	dead	dead n.d.	dead		dead n.d.
		3rd day after labor	—	—	—	dead n.d.	dead n.d.	dead	dead		dead
			—	—	—	dead	dead	dead	dead		dead
		7th day after labor	—	—	—	—	dead	dead	reaction	dead	dead
			—	—	—	—	dead	dead	dead	dead	dead
	M. I.	Before labor	—	dead n.d.	dead n.d.	dead	reaction	dead			dead
		During attack	—	dead	dead	dead	dead				dead n.d.
		5th day after labor	—	—	—	—	—	dead	dead	dead	dead
			—	—	—	—	dead n.d.	dead	dead	dead	dead
		9th day after labor	—	—	—	dead n.d.	dead	dead	dead	dead	dead
			—	—	—	—	reaction	dead	dead	dead	dead
	H. Y.	Just after labor	—	—	dead	dead	reaction	dead			dead
		During attack	—	reaction	dead	dead	dead	dead			dead
		$3\frac{1}{2}$ days after labor	—	—	—	dead n.d.	reaction	reaction	dead	dead	dead
			—	—	—	dead	dead	dead	dead	dead	dead

Eclamptic women	S. Y.	Before labor	—	dead n.d.	dead n.d.	dead n.d.	dead	dead			dead
		During attack	—	dead n.d.	dead n.d.	dead	dead	dead			dead
		4th day after labor	—	—	—	—	dead	dead	dead	dead	dead
			—	—	—	—	dead	dead	dead	dead	dead
		24th day after labor	—	—	—	—	—	dead	reaction	reaction	dead
			—	—	—	—	—	dead	reaction	dead	dead
	T. Y.	Just after labor	—	reaction	—	dead	dead	reaction	reaction	dead	dead
		During attack	—	dead n.d.	dead	dead	—	dead	dead	dead	dead
		9th day after labor	—	—	—	—	—	dead	dead	dead	dead
			—	—	—	—	—	dead	dead	dead	dead
	R. Y.	Before labor	dead	dead	dead n.d.	dead	dead n.d.	dead			dead
		During attack	—	dead	dead	dead n.d.		dead			dead
		$3\frac{1}{2}$ days after labor	—	—	—	reaction	dead	dead			dead
			—	—	—	—	dead	dead			dead
		$6\frac{1}{2}$ days after labor	—	—	—	dead n.d.	dead n.d.	dead			dead
			—	—	—	—	—	dead			dead

TABLE 3—*Continued*

	NAMES OF PERSONS FROM WHOM BLOOD SERUM HAD BEEN TAKEN FOR THE EXPERIMENTS	EXPERIMENTAL FLUID....	I	II	III	IV	V	VI	VII	VIII	IX	CONTROL
		SERUM.....	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0.05	0.025	—
		PHYSIOLOGICAL SALT SOLUTION.....	0.3	0.4	0.5	0.6	0.7	0.8	0.9	0.95	0.975	1.0
		PLACENTAL EXTRACT.....	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Eclamptic patients	S. T.	Before labor		—	dead	dead	dead	dead	dead	dead	dead	dead
		During attack		—	dead	dead	dead	dead	dead	dead	dead	dead
		11th day after labor		—	—	—	—	dead	dead	dead	dead	dead
				—	—	—	—	dead	dead	dead	dead	dead
	T. M.	Before labor	—	—	dead	dead	dead	dead	dead n.d.	dead n.d.		dead
		During attack	—	—	dead	dead n.d.	dead n.d.	dead	dead n.d.	dead		dead n.d.
		3rd day after labor		—	—	—	dead n.d.	dead	dead n.d.	dead		dead
				—	—	—	dead	dead	dead	dead		dead
		7th day after labor		—	—	—	—	dead	dead	reaction	dead	dead
				—	—	—	—	dead	dead	dead	dead	dead
	M. I.	Before labor	—	—	dead n.d.	dead n.d.	dead	reaction				dead
		During attack	—	—	dead	dead	dead	dead	dead			dead n.d.
		5th day after labor	—	—	—	—	—	—	dead	dead	dead	dead
			—	—	—	—	—	dead n.d.	dead	dead	dead	dead
		9th day after labor	—	—	—	—	dead n.d.	dead	dead	dead	dead	dead
			—	—	—	—	—	reaction	dead	dead	dead	dead
	H. Y.	Just after labor	—	—	—	dead	dead	reaction	dead			dead
		During attack	—	—	reaction	dead	dead	dead	dead			dead
		$3\frac{1}{2}$ days after labor	—	—	—	—	dead n.d.	reaction	reaction	dead	dead	dead
			—	—	—	—	dead	dead	dead	dead	dead	dead

Eclamptic patients	S. Y.	Before labor	—	—	dead n.d.	dead n.d.	dead n.d.	dead	dead			dead
		During attack	—	dead n.d.	dead n.d.	dead n.d.	dead	dead	dead			dead
		4th day after labor			—	—	—	dead	dead	dead	dead	dead
					—	—	—	dead	dead	dead	dead	dead
		24th day after labor			—	—	—	—	dead	reaction	reaction	dead
					—	—	—	—	dead	reaction	dead	dead
	T. Y.	Just after labor	—	—	reaction	—	dead	dead	reaction	reaction	dead	dead
		During attack	—	—	dead n.d.	dead	dead		dead	dead	dead	dead
		9th day after labor			—	—	—	—	dead	dead	dead	dead
					—	—	—	—	dead	dead	dead	dead
	R. Y.	Before labor	—	dead	dead	dead	dead	dead				dead
		During attack	dead	dead	dead n.d.	dead n.d.	dead n.d.	dead				reaction
		$3\frac{1}{2}$ days after labor	—	—	—	—	dead	dead				dead
			—	—	—	reaction	dead	dead				dead
		$6\frac{1}{2}$ days after labor	—	—	—	—	—	dead				dead
			—	—	—	dead n.d.	dead n.d.	dead				dead
	R. S.	Before labor	—	—	—	dead	dead	dead	dead	dead		dead
		During attack	—	—	—	dead	dead	dead	dead	dead		dead
		24th hours after labor			—	—	—	—	dead	dead		dead
					—	—	—	reaction	dead	dead		dead
		5th day after labor				—	—	—	—	dead	dead	dead
						—	—	—	reaction	dead	dead	dead
	A. Y.	During attack	dead	dead n.d.	dead	dead			dead			dead
			—	dead n.d.	dead	—			dead			dead
		5th day after labor		—	—	dead	dead n.d.	dead n.d.				dead

TABLE 3—*Continued*

	NAME OF PERSON FROM WHOM BLOOD SERUM HAD BEEN TAKEN FOR THE EXPERIMENT	EXPERIMENTAL FLUIDS...	I	II	III	IV	V	VI	VII	VIII	IX	CONTROL
		SERUM.....	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0.05	0.025	—
		PHYSIOLOGICAL SALT SOLUTION.....	0.3	0.4	0.5	0.6	0.7	0.8	0.9	0.95	0.975	1.0
		PLACENTAL EXTRACT....	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Eclamptic patients	N. S.	Before labor	—	reaction	dead	dead	dead	dead				dead
		During attack	—	reaction	dead	dead	dead	dead				dead
	S. Y.	During attack	reaction	dead	dead	dead				dead	dead	dead
			dead	dead n.d.	dead	dead				dead	dead	dead
	Y. S.	Just before labor	—	dead	dead	dead	dead	dead				dead
		During attack	—	dead	dead	dead	dead	dead				dead
		44th hours after labor		—	—	—	—	dead	dead			dead
				—	—	—	—	dead	dead			dead
	T. N.	Just before labor	dead	dead	dead	dead	dead	dead	dead			dead
		During attack	dead	dead	reaction	dead	reaction	dead	dead			dead
		2nd day after labor			—	—	—	—	dead	dead		dead
					—	—	—	—	reaction	dead		dead
		8th day after labor					—	—	dead	dead		dead
							—	—	dead	reaction		dead

TABLE 4

ANIMALS EMPLOYED IN THE EXPERIMENT	EXPERIMENTAL FLUIDS.. SERUM / PHYSIOLOGICAL SALT SOLUTION / PLACENTAL EXTRACT....	I 0.6 0.4 1.0	II 0.5 0.5 1.0	III 0.4 0.6 1.0	IV 0.3 0 7 1.0	V 0.2 0.8 1.0	VI 0 1 0.9 1.0	Control — 1.0 1.0
Rabbit 59 ♂	Before convulsion	—	—	dead	dead			dead
		—	—	dead	dead			dead
	After convulsion	—	—	reaction	dead			dead
		—	—	dead	dead			dead
Rabbit 60 ♀	Before convulsion	—	—	—	dead	dead	dead	dead
		—	—	—	dead	dead	dead	dead
	After convulsion	—	—	—	dead	dead	dead	dead
		—	—	—	dead	dead	dead	dead
Rabbit 61 ♀	Before convulsion	—	—	—	dead	dead		dead
		—	—	—	dead	dead		dead
	After convulsion	—	—	—	dead	dead		dead
		—	—	—	dead	dead		dead

TABLE 5

ANIMALS EMPLOYED IN THE EXPERIMENT	DATE OF INJECTION	BODY WEIGHT OF ANIMALS	DOSE OF PLACENTAL EXTRACT INJECTED	RESULT
		grams	*cc.*	
Rabbit 4. ♀	19th August 1916	1800	7.8	No reaction
	22d August 1916	1710	2.0	No reaction
	26th August 1916	1700	3.0	No reaction
	30th August 1916	1800	4.0	Convulsion, dead
Rabbit 5. ♂	19th August 1916	1190	1.2	
	22d August 1916	1220	1.3	
	26th August 1916	1230	2.0	No reaction
	30th August 1916	1230	2.5	Convulsion, dead
Rabbit 10. ♀	19th August 1916	1480	1.0	No reaction
	22d August 1916	1450	1.2	No reaction
	26th August 1916	1310	1.6	No reaction
	30th August 1916	1220	2.0	Convulsion, dead

COMPARISON OF THE PATHOLOGICAL ANATOMY OF ANIMALS KILLED WITH PLACENTAL EXTRACT AND THAT OT ECLAMPSIA

It is important to the present thesis to show an agreement between the anatomical changes of animals dead after the injection of placental extract and those observed in eclampsia.

Two rabbits and two dancing mice were killed with a heavy dose of the placental extract. The gross and microscopic examination of the dead animals showed; a decrease in the coagulability of the blood, hemorrhages in the lungs on both sides and often parenchymatous hemorrhage in the liver as well as the frequent formation of thromboses in both the lungs and the liver, these thrombi consisting chiefly of blood-platelets mixed with blood shadows.

Both the hemorrhages and the thromboses in the animals are in agreement with the anatomical features of eclampsia, but the decreased coagulability of the blood appears to be a discordant finding. It must be borne in mind, however, that decreased coagulability of the blood is generally noted in death by acute intoxication. Hemorrhage in the viscera is also to be regarded as a usual occurrence during convulsions. The formation of thrombi is reported by the authors who have studied the effects of visceral extracts.

Notwithstanding the parallelism just described, the experiment is not quite free from objection because the results spoken of above have been obtained in animals killed with a single injection. A more exact experimental counterpart of the conditions supposed, under our theory, to exist in eclampsia would be produced by exposing the animals to a more prolonged action of placental extract. Such condition was established in the following experiment.

Sublethal doses of the extract were injected into rabbits three times daily over a period of seven to twelve days; the animals being finally killed with a lethal dose. In 5 of the 7 rabbits thus treated there was a marked increase in the coagulability of the blood; in the other two animals the coagulability of the blood was diminished. It happens that in the latter

two animals death was produced with several heavy doses of extract injected at intervals of two to five minutes. It is possible, therefore, that the diminished coagulability of the blood in these animals was the result of this extraordinary treatment.

In 3 of these animals extraperitoneal hemorrhage was found along the ileopsoas muscle; in 2 of these the hemorrhage was quite recent, while in the third animal it was evidently of considerable duration. In 1 animal striking edema was found in the subcutaneous tissues of the whole body and in the muscles; ascites was also present.

In 6 animals the alveoli of the lungs and the mucosa of the bronchioles exhibited hemorrhages; macroscopically petechiae could be seen on the surface of the lungs. Thromboses were found in the lungs, some of which were found to be layered, some undergoing a partial hyaline degeneration and some being actually organized.

Fatty degeneration was demonstrable in the liver of all of the animals employed in the experiments; in those injected during three days this change was still slight, but in the animals in which the injections had been extended over seven to twelve days the degeneration was striking. Portions of the liver, as was seen in 3 of the animals, had undergone necrosis and in these Glisson's sheath presented a perivascular infiltration.

The kidney presented cloudy swelling in 5 instances, in some of which the renal cells had undergone vacuolar degeneration; others presented a slight fatty degeneration and still others showed hemorrhage in the medullary portion.

In most instances, no change was found in the heart; a single case, however, presented edema and a slight fatty degeneration. In the spleen there was recognizable passive congestion, often with pigment deposits.

In the literature on the pathological anatomy of eclampsia we find, first of all, the results arrived at by Schmorl (9), who gives the following features, based on a study of 73 cases. In almost all cases anaemic and hemorrhagic necrosis was seen in the liver and thromboses and hemorrhage were found in

the lungs. In the heart fatty degeneration of the cardiac muscle was frequently encountered and hemorrhage and destruction of the muscle elements were, also, not infrequent. In the kidneys cloudy swelling of the secreting cells and fatty degeneration were usually found and necrosis and thrombosis were, also, often observed.

Peterson (10) pointed out, in the liver, hemorrhage, thromboses and intensive fatty degeneration and he detected, in the kidneys, coagulated material in the interior of the urinary tubules and hemorrhage, for the greater part, confined to the medullary portion.

These pathological findings have been generally confirmed. The coagulability of the blood in eclampsia has been found to be frequently, though not always increased.

From the preceding descriptions an agreement is apparent in the pathology of eclampsia and that of the animals killed with placental extract excepting the slighter alterations met with in the kidneys of the latter.

I do not assume that the changes referred to are characteristic of intoxication with placental extract and of eclampsia only; indeed, I am aware that similar changes may be brought about, also, with extracts from other viscera. I wish only to point out the fact that pathological changes similar to those seen in eclampsia are producible in animals with placental extracts.

CONCLUSIONS

The present investigation has shown that in its capacity to neutralize the poisonous action of placental extract the serum of eclamptic women is much inferior to that of normal individuals, whether male or female and that the normal capacity of the serum, in this respect, is restored, in eclamptic women, on the fourth or fifth day after labor.

The investigation shows, also, that this abnormality of the serum in eclampsia is not brought about by the convulsion itself.

Furthermore, not only has a marked resemblance been pointed out between the symptoms produced with the placental ex-

tract and those of the eclamptic attack, but an almost perfect agreement has been found between the anatomical features of eclampsia and those of the animals which were killed by repeated injections of placental extract.

From these facts we feel justified in drawing the conclusion that *the true nature of eclampsia is nothing other than an intoxication by the placental poison which is made possible by a weakening in its normal capacity of neutralization on the part of the maternal blood.*

The clinical symptoms as well as the anatomical alterations mentioned above are, of course, not peculiar to intoxication by placental extract, as has been shown by several authors (Dold and Ogata (2), Kinoshita (42), Takeuchi (43). However, the derivation of the injurious agents, in eclampsia, from the placenta seems indicated from the remarkable fact that the symptoms cease when the placenta has been eliminated. It is true that eclampsia does rarely occur after the discharge of the placenta, but not after a period of twenty-four hours following the placental discharge. Even for this phenomenon, however, there is an analogy in the animal experiments with the placental extracts in those instances in which the symptoms developed several hours after the injection of the extract, or, as it rarely happened, when a second paroxysm occurred as late as ten hours following the injection. It may also be suggested that this later occurrence of eclampsia may be the symptom complex of intoxication by a poison produced by autolysis of the uterus itself as a part of the process of involution of that organ. This idea is rendered plausible by the fact, shown by Yoshimura (44), that uterus extracts contain a poison.

While the foregoing investigation leaves little doubt as to the nature of eclampsia, one question still remains open; namely, what causes the weakening of the neutralizing capacity of eclamptic serum? Our work on this question is still in progress.

HISTORICAL REVIEW OF THE THEORIES OF ECLAMPSIA

Eclampsia had early been looked upon as a uraemia (Lever, Frerichs, Cohnheim, Spiegelberg), but these two conditions were found to be different not only as to clinical symptoms, but also with respect to the blood findings. According to Szili (14), the freezing point of the serum of eclamptic women can not be distinguished from that of the serum of normal individuals; furthermore, an accumulation of urinary products is not demonstrable in the blood of eclamptic women.

Halbartsma and Loehlein ascribed eclamptic convulsions to a reflex effect of pressure in the pelvis of the kidney. They supposed that such pressure was produced by a compression of the ureters at the pelvic brim by the head of the foetus. This view could not be maintained because of the fact that the eclamptic convulsions take place, in most cases, during the pregnancy or at the beginning of labor; that is, at a time when the head of the foetus is not yet in position to compress the ureters.

Eclampsia has been assumed to be caused by bacteria (Doléris, Poncy, Blanc, Mueller, Albert). Gerdes isolated from the viscera and from the placental surface of the uterus of eclamptic individuals, an organism, which he named *Bacillus eclamptica*. This organism, however, was identified by Hofmeister as the proteus vulgarus.

Zweifel ascribes eclampsia to an intoxication by lactic acid which he found in the urine and serum of eclamptic women and he gives examples of increased lactic acid in gravidae suffering from nephritis as evidence that the increase of lactic acid is not an effect of the convulsions. This view seems incompatible with the fact that an increased quantity of lactic acid is found in women suffering, not from eclampsia, but from nephritis.

Similar to these views of uraemic or lactic acid intoxication in eclampsia are the views of Stumpf and of Landois according to which eclampsia is due respectively to aceton and creatinin poisoning. Both of these views are lacking in proof.

Bouchard and his school advanced the theory that eclampsia is an autointoxication caused by metabolic products. This theory has received support from the later experiments of Chambrelent, who reported that the serum of eclamptic women is three to four times as toxic as normal serum.

According to the so-called hepato-toxhaemic theory, poisonous products of metabolism are thoroughly neutralized when the liver is normal, but autointoxication is caused by these poisonous products when the function of the liver is disturbed. The poison in such case is regarded by Massin as a leucomaine and by Ludwig and Savor as carbamic acid. This view was denied by Volhard and Schuhmacher who found that the serum of eclamptic women is not more toxic then normal serum—a conclusion that is confirmed by the present investigation.

According to Kollmann and Dienst, eclampsia is a globulin intoxication resulting from imperfect function of the kidney; these authors detected an enormous increase of the fibrin content of the blood of eclamptic women and they believe, this to be due to an accumulation of globulin. This excess of globulin the authors assume to be derived from the foetus being retained in the maternal circulation because of an imperfect function of the kidney. Dienst maintains this view in some more recent publications. According to this view, also, the serum of eclamptic women ought to be more toxic than normal serum, a requirement not met by the facts. Against this theory stand, furthermore, the instances of eclampsia in the absence of a foetus, as in two cases of the author of eclampsia with hydatidiform mole and other similar instances in the literature.

Lamsbach (25) collected from the literature 68 cases in which eclampsia occurred after the death of the foetus. In 50 of these cases the foetus was found macerated; in 9 cases the foetus was well preserved, while in the remaining cases there was a hydatidiform mole. In view of these facts a theory seeking the cause of eclampsia in the foetus can not be justified.

The same objection must be made to the idea of Kinoshita and his collaborators, who produced death in animals after

symptoms resembling those of eclampsia by injecting the albumin-free extract of the animal foetus. The toxic effect thus produced is ascribed by the authors to a substance named by them "eclampsin."

A number of different views assume the placenta to be the source of the condition of eclampsia.

Having shown that syncytial cells actually gain access to the maternal circulation, Veit (27) carried out the following experiment: fluid expressed from the placenta was injected into the abdominal cavity of a rabbit, which then developed albuminuria. Veit observed, furthermore, that the serum of such a rabbit acquired the property of dissolving the syncytial cells and he assumed this action to be due to an immunological reaction product, which he designated "syncytiolysin." On the basis of these observations this author assumed that eclampsia is due to the rapid extrance into the maternal blood of syncytial cells in a quantity greatly in excess of the available syncytiolysin that has developed during the pregnancy. The albuminuria occurs, according to Veit, when the syncytial cells gain access to the maternal circulation more slowly and in more moderate excess of the available syncytiolysin.

If this assumption is correct, the serum of the pregnant woman ought to show, with respect to the poisonous property of placental extract, a stronger neutralizing capacity than does that of a man or a non-pregnant woman and experimental animals ought to be made immune, by the injection of placental extract, requirements that are not fulfilled in the present investigation.

Ascoli (28) agrees with Veit in the assumption of a poisonous action of syncytial elements, but his experiment in which serum was injected into the submeningeal spaces, does not appear to be convincing. Wormser (29) repeated that experiment with negative result.

Liepmann (30) looks upon eclampsia as an intoxication of placental origin, basing his belief on experiments carried out as follows: a salt solution emulsion of the powder made from the placenta was injected into the abdominal cavity of rabbits.

The emulsion from placentae of eclamptic women was especially toxic. This result is not confirmed in the present investigation.

Weichardt (31) assumes that antibodies against placental cells appear in the maternal blood-stream, when the cells gain access to it and these antibodies dissolve the cells, which then liberate a toxin called by the author "syncytiotoxin." In the ordinary pregnancy the blood possesses, furthermore, an antitoxic mechanism, which, according to this author, is disturbed in eclampsia. The present study has provided strong support for this theory, inasmuch as it demonstrates, for the first time, a distinct difference in the capacity of normal serum and that of eclamptic women, to neutralize the toxic property of placental extract.

In a study with Pilz (32) Weichardt comes to the conclusion that placental pulp contains two different toxic principles, one of which accelerates the coagulation of the blood while the other attacks the respiratory center.

According to Hofbauer (34), eclampsia is an intoxication brought about by a substance derived from the liver by a process of autolysis under the influence of a ferment produced in the placenta and discharged into the maternal blood. According to this idea the serum of eclamptic individuals should be more toxic that that of normal persons. However, the present investigation shows that no such difference is demonstrable.

Finally, there is the anaphylaxis theory of eclampsia. A considerable number of observers, among whom may be mentioned Rosenau and Anderson (35), Thies (36) and Lockemann (37), Graefenberg (38) and Bauereisen (39), believe that anaphylaxis is induced, during pregnancy, by foetal serum, placenta or amniotic fluid. However, a careful examination of the experimental basis of this theory discloses evident occasion for doubt as to its correctness.

Fellaender (40) and Guggisberg (41) deny the anaphylactic nature of eclampsia and in some unpublished research of my own I have reached the same negative conclusion.

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THE PRODUCTION OF ANTI-HUMAN HAEMOLYSIN

EDWARD B. VEDDER

Colonel Medical Corps, United States Army

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In the performance of the complement fixation test for syphilis,[1] the human haemolytic system has a great advantage over the very generally used sheep haemolytic system in avoiding the fallacy of natural anti-sheep haemolysin which is present in a large proportion of human sera. The greatest objection to the use of the human system has been the difficulty in immunizing rabbits against human cells, because the majority of the rabbits immunized by the usual methods die before producing a haemolytic serum of sufficiently high titre. The necessity of producing anti-human haemolysin on a large scale led to the study of a method whereby this difficulty could be avoided.

It has been very generally assumed that rabbits immunized against human red corpuscles died of anaphylaxis. This undoubtedly may occur, but apparently the usual cause of death of such rabbits is embolism, the emboli being formed from the masses of agglutinated red cells. This was strongly suggested because the symptoms and manner of death after injections of red cells were practically identical with the symptoms manifested by rabbits that had received an intravenous injection of a concentrated solution of arsphenamine; and Danysz (1) has shown that such animals die from the effects of emboli formed by the precipitated arsphenamine.

[1]It is believed that the use of the term "Wassermann reaction" should be discontinued. The test is really an application of the Bordet-Gengou reaction, and Wassermann's ideas as to the specificity of the luetic antigen have been discarded long ago, together with most of the technique described by him.

Investigation showed that the serum of normal rabbits does not often agglutinate all four types of red cells (Moss' classification) and that the agglutinations are weak when they do occur. But as early as six days after the first immunizing dose of red cells, the serum as a rule, strongly agglutinates all four types of cells. It is evident therefore that as the result of the immunizing injections agglutinins are produced in considerable amount long before the production of haemolysin. In order to determine whether rabbits were killed by agglutination, a sensitized rabbit was given an injection of cells that could not agglutinate. The experiment was performed as follows:

A rabbit was selected that had had three injections of human erythrocytes. On receiving the last dose he was very sick and a companion rabbit died. Five days later his serum was tested and found to agglutinate human red cells strongly. The rabbit was then bled, and 10 cc. of his serum was diluted to 40 cc. with physiological salt solution. This mixture was heated for one hour between 78–80°C. to destroy agglutinins and produce agglutinoids.

Freshly washed human erythrocytes were then sensitized with this heated serum for twelve hours in the ice box in the proportion of one part packed cells to eight parts of serum. The cells were then centrifuged off, washed, and tested with the rabbit's fresh serum. The cells no longer agglutinated, presumably being saturated with agglutinoids. Four cubic centimeters of these packed cells that could not be agglutinated were injected into the highly sensitized rabbit. The rabbit bore the injection well though experience indicated that a smaller quantity of fresh untreated cells would certainly have killed the rabbit.

The first method of producing haemolysin here described was designed simply to avoid causing the death of rabbits by agglutination of the injected cells. Large healthy male rabbits were selected, and their serum tested against human cells. If agglutination was absent or slight, they were given an intravenous injection of 4 cc. of closely packed human erythrocytes on three successive days. This does not appear to injure the rabbit and the great bulk of the material is thus injected prior to the formation of agglutinins. The rabbits are then kept

without treatment and well fed for from two to three weeks. Then they are given very slowly, daily intravenous injections of 0.5 cc. of packed cells until five or six injections have been given. The titer of the serum of rabbits so treated rises very promptly as the result of these small injections, and a satisfactory haemolysin may be obtained by this method in the majority of the rabbits so treated. The fact that immune substances can be called forth in large amount by such small injections in a previously sensitized animal may be of some importance in other methods of immunization.

An endeavor was next made to determine which fraction of the red cell was responsible for the production of haemolysin, and to immunize rabbits with that fraction. It was readily determined that haemolysin does not unite with haemoglobin, but does unite with the stroma of the corpuscle. Thus when a suspension of stroma is mixed with haemolysin, and incubated, and subsequently the stroma is centrifuged off, washed and resuspended in salt solution, if complement be added the stroma will all dissolve. On the other hand if the fluid remaining after the stroma is centrifuged out be tested by adding complement and fresh corpuscles, no haemolysis will occur, thus proving that all the haemolysin was removed with the stroma. This indicates not only that the haemolysin has united with the stroma, but that the solution of the stroma is the important factor in haemolysis, and that the setting free of the haemoglobin is purely incidental. From this it appeared that if rabbits were immunized with the stroma of red corpuscles, they should produce haemolysin.

Difficulties were encountered in the production of stroma. If the blood is laked with an excess of distilled water, the haemoglobin is set free and the stroma may be recovered by centrifugation for some time at high speed. But only a small part of the total amount of stroma is recovered by this method, for a great part of the stroma goes in solution in the water. Furthermore the stroma so obtained is distinctly toxic to rabbits. Mathews (2) has stated that stroma freed from its haemoglobin behaves as a poison causing intravascular coagulation,

although the haemoglobin-stroma compound as it exists in the corpuscle is inert in this respect. At any rate, stroma prepared as above described was too toxic for use, as 0.5 cc. of packed stroma and all larger amounts killed the rabbits when injected intravenously. No satisfactory method of preparing stroma was found in the literature, and after several efforts the following method was used and has given good results.

Preparation of stroma. Fresh red corpuscles are washed in salt solution and packed in the centrifuge the exact amount being noted. The corpuscles are diluted with an equal volume of 0.85 per cent NaCl solution in a cylinder and placed outside the window where ordinary illuminating gas is allowed to bubble through it freely for fifteen to twenty minutes. At the end of this time the corpuscles will have taken on the characteristic cherry red color. The salt solution is centrifuged off and sufficient distilled water is added to produce haemolysis. This mixture should be left for an hour or so to permit all the haemoglobin to become dissolved out the stroma then being separated by centrifugation at high speed. About 33 per cent of the volume of the corpuscles should be recovered as yellowish white stroma. The outline of the stroma cells so obtained is perfect under the microscope, and it appeared that by this method the whole stroma is obtained. The carbon monoxide appears to preserve the stroma so that it does not go into solution so readily when washed. This effect of illuminating gas was discovered by accident. The stroma so obtained is diluted with salt solution up to the original volume of the packed corpuscles, and this suspension is used for immunizing rabbits.

It was found that 2.5 cc. of this suspension of stroma per kilo of body weight constituted the minimal lethal dose, and haemolysins of a satisfactory titer were readily produced as follows. A first intravenous injection of 1 cc. was given, followed at five-day intervals by doses of 2 cc. These doses do not harm the rabbits, and haemolysins have been prepared in the majority of rabbits so immunized. Three such injections have proved sufficient to give haemolysin of good titer in the short period of fifteen days in several rabbits. The haemolysin prepared by the injection of stroma has been used in duplicate tests in the complement fixation for syphilis, using

haemolysin prepared in the usual way in the control. In about one hundred such tests, no differences in reaction were recorded, and haemolysin prepared from stroma appears to be suitable for this purpose.

An attempt was now made to determine which fraction of the stroma was responsible for the production of haemolysin. Bang and Forssman (3) claimed to have produced haemolysin by the injection of the lipoids obtained in the ether extract of corpuscles. However I have been totally unable to produce a trace of amboceptor or haemolysin by the injection of the ether extract of corpuscles. A number of such experiments were performed with extracts prepared in various ways. The following is an illustration:

Thirty cubic centimeters of packed cells were evaporated to dryness and extracted with 150 cc. of ether in successive portions in a shaking machine. The ether extract was evaporated to 30 cc. so that 1 cc. of the extract represented the lipoids extracted from 1 cc. of corpuscles. Before administration this was evaporated to dryness, and the residue taken up with a minimal amount of alcohol, and this was emulsified in salt solution. Five or six injections at intervals of five days and in amounts representing the lipoids from 2 cc. of corpuscles at each injection failed to produce a trace of haemolysin. Furthermore it can be readily shown that haemolysin does not combine with this lipoid extract, whereas it does combine with the proteid fraction of the stroma.

FRACTIONATING THE STROMA

Ten cubic centimeters of stroma corresponding to 10 cc. of packed cells were dissolved by the addition of a few drops of decinormal NaOH. The yellowish brown clear solution was carefully neutralized with acetic acid, and when the neutral point was reached, the globulins precipitated out. The globulins were centrifuged off, washed in distilled water, and then dissolved in salt solution rendered slightly alkaline with NaOH. Rabbits injected with this solution totally failed to

produce haemolysin. Five injections at five-day intervals were given.

The supernatant solution represented albumins with the lipoids. It coagulated on boiling, and showed no trace of globulin on half saturation with ammonium sulphate. Two rabbits injected with this solution produced haemolysin, one of them in sufficient titer for use. Rabbits injected with stroma previously extracted with ether and freed from lipoid, also produced haemolysin. In all of these experiments the solutions were freshly prepared just prior to administration.

SUMMARY

Anti-human haemolysin may be produced readily by the injection of stroma. Stroma may be more easily prepared by first saturating the cells with illuminating gas. The lipoid fraction of the cell is totally ineffective. The proteid fraction of the stroma unites with haemolysin, and on injection produces haemolysin. The globulin fraction of the stroma failed to produce haemolysin, whereas the proteid fraction from which all globulins were removed did produce haemolysin.

DISCUSSION

It has been shown by others and I have also found that the haemolysin is either a globulin or is precipitated with the globulins; more probably the former hypothesis is correct for the total haemolytic power of a serum is contained in the globulins whether precipitated out by salting or by dialysis. In this case therefore, an antigen consisting of a specific albumin produces an antibody consisting of a specific globulin.

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THE BACTERICIDAL ACTION OF THE WHOLE BLOOD OF RABBITS FOLLOWING INOCULATIONS OF PNEUMOCOCCUS BACTERINS[1]

GEORGE D. HEIST AND SOLOMON SOLIS-COHEN

From the Jules E. Mastbaum Research Laboratory of the Jewish Hospital, Philadelphia

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The Journal of Immunology, volume 3, number 4, contains an account of our earlier studies of the bactericidal action of whole, uncoagulated blood on pneumococci. In the same paper, in association with Myer Solis-Cohen, we described a similar bactericidal action of certain whole bloods upon the globoid bodies of acute anterior poliomyelitis, and Matsunami and Kolmer (2), using our method, found that the blood of certain animals was bactericidal for meningococci. In our first paper we gave the details of the method by which we were able to bring the blood, before it had time to coagulate, into contact with bacteria.

The essential feature of the method, suggested by Benjamin F. Lacy, is that a capillary glass tube be filled, by capillary attraction, up to a fixed mark, with broth culture of pneumococci, and then emptied. A certain number of pneumococci remain sticking to the wall of the tube. Blood as it comes from capillary or vein is allowed to flow up the tube to the mark and the tube is then sealed and incubated. If the blood has no bactericidal action the pneumococci which have remained on the wall of the tube find themselves in a favorable medium and multiply rapidly: if it is bactericidal they are killed and no growth results. Readings are made by blowing out the contents of the tube on a glass slide, staining and examining them

[1] The research was made possible through the kindness of Mr. Samuel S. Fels. Read before the Congress of American Physicians and Surgeons, Atlantic City, N. J., June 17, 1919.

under the microscope. By combining several capillary tubes into one many-stemmed pipette, modelled after the one Wright (6) uses for estimating the coagulation time of the blood, and by using a series of ascending dilutions of broth culture, an approximate quantitative value may be given to the test.

Using this method we were able to show that the blood of pigeons, a species immune to pneumococcal infection, destroys virulent pneumococci *in vitro*; whereas the normal blood of rabbits, a species highly susceptible to this organism, has no such action. If, however, rabbits whose blood, previously tested, proved a favorable culture medium for the germs, were suitably immunized with dead bacteria, their blood would show an acquired bactericidal power. No bactericidal action on pneumococci was, however, found in the defibrinated blood or in the serum of either pigeon, normal rabbit or inoculated rabbit.

Contaminations by non-pathogenic bacteria were rarely met with in testing the bactericidal activity of whole blood. So far as our observations went, non-pathogenic bacteria failed to grow in whole blood unless comparatively large numbers were seeded. We advanced the hypothesis that only those bacteria grow, *in vitro*, in whole uncoagulated blood, which are pathogenic for the animal from which the blood was taken. Our later observations give confirmation to this hypothesis.

The experiments on the bactericidal powers of whole blood have been continued. The immediate and practical object has been to find out whether the test can be made use of in determining the efficiency of prophylactic inoculations of pneumococci, especially of pneumococci belonging to type III. Much effort has been expended in the past to find a test which will do this. Agglutination has proved the most reliable method but the agglutination test does not record slight degrees of immunity, especially of immunity to type III pneumococci. We shall often refer to the agglutination test, using it as a standard of comparison for the test of the bactericidal action of whole blood. The mouse protection test has a field in which its value is unquestioned, but the time, labor, and expense con-

sumed in the performance of a single mouse-protection test are so great that it is not practical when one has to examine a large number of men or animals.

The work here reported may be grouped under two headings. The first concerns the virulence of the pneumococci and the relation such virulence bears to their ability to grow in whole blood. The second concerns the antibodies present in the blood to be examined and their action on the pneumococci.

I. VIRULENCE AND ITS RELATION TO ABILITY TO GROW IN BLOOD

In agglutination tests on pneumococci the virulence of the strain from which the agglutinating fluid is made is not of essential importance, but in testing the bactericidal power of whole blood the virulence of the pneumococci used is most important. When small numbers of strictly non-pathogenic bacteria, such as *B. subtilis*, are seeded in whole blood they fail to grow. Similarly, pneumococci which have lost their power of killing rabbits fail to grow in rabbit blood. Therefore, in order to test the bactericidal qualities of the blood of inoculated rabbits it is necessary that we make use of a strain of pneumococci which will grow readily in the blood of normal rabbits, in other words, a strain which is virulent for rabbits. It is further necessary that this ability to grow in normal blood shall remain fairly constant. In experimentation upon animals it is easy to fulfill these two conditions. By passage through susceptible animals we may increase the virulence of a strain of pneumococci, and maintain it at its maximum with little fluctuation, and in this way secure a fixed point against which to test the antibody content of the blood under examination.

The mere fact that a-virulent pneumococci fail to grow in the blood of normal rabbits is a negative phenomenon which suggests the question: Is an increase of virulence accompanied by an increased ability to grow in blood? Our first experiment was carried out to answer this question.

We had on hand a culture of type III pneumococci, obtained from The Rockefeller Institute some months previous and referred to as A66. It was moderately virulent for mice but

not for rabbits. Three rabbits were injected intraperitoneally with this strain. One received 1 cc., one 5 cc., and one the pneumococci centrifuged from 500 cc. of twenty-four hour broth culture. All three rabbits remained well. A test of the ability of this strain to grow in whole blood was made on 5 normal rabbits. The results of this test are shown in table 1. The medium used for growing the pneumococci for this test, as well as for all the tests recorded in this paper (with one exception which is noted) was beef infusion broth, neutral to plus 0.2 with phenolphthalein. It was sterilized in an Arnold sterilizer and 1 per cent of glucose and a few drops of sterile defibrinated rabbit's blood added to each tube. The period of incubation is noted before each test. It was found most satisfactory to use a young culture, from twelve to eighteen hours old.

TABLE 1

The growth of type III pneumococci, avirulent for rabbits, in the whole blood of normal rabbits

DILUTIONS OF 12 HOUR CULTURE	RABBITS				
	1	2	3	4	5
Undiluted	−	+	+	+	+
1:4	+	+	+	+	−
1:16	−	−	+	−	+
1:64	−	−	−	−	−
1:256	−	−	−	−	−

In this and in subsequent tables, each sign represents the results in one capillary tube which was seeded from the dilution indicated and then filled with blood in the manner described on page 1. *Plus* indicates that the pneumococci grew, *minus* that they did not. Many trials have shown that when no pneumococci can be found microscopically after twenty-four hours incubation the tube is sterile, and if its contents be blown out into a tube of broth no growth results.

The ability of this strain of type III to grow in rabbit blood as well as its virulence for rabbits is seen to be low. This fact having been determined the strain was then passed through 7 mice successively. Each time the pneumococci were recovered

from the heart of the mouse. A portion of this passage culture was injected into the next mouse and at intervals the ability of the pneumococci to grow in rabbit blood was tested. The progressive increase of the virulence of the strain for mice was accompanied by like increase in its ability to grow in rabbit blood. The passages and the tests upon the blood of rabbits are conveniently combined in table 2.

TABLE 2

Increasing ability of type III pneumococci to grow in rabbit blood as the strain is passed through mice

Passage through mouse 1	
Passage through mouse 2	
DILUTIONS OF 24 HOUR CULTURE FROM HEART OF MOUSE 2	NORMAL RABBIT
1:10	+
1:100	+
1:1,000	−
Passage through mouse 3	
DILUTIONS OF 24 HOUR CULTURE FROM HEART OF MOUSE 3	NORMAL RABBIT
1:10	+
1:100	+
1:1,000	−
1:10,000	+
Passage through mouse 4	
DILUTIONS OF 24 HOUR CULTURE FROM HEART OF MOUSE 4	NORMAL RABBIT
1:10	+
1:100	+
1:1,000	+
1:10,000	−
Passage through mouse 5	
DILUTIONS OF 24 HOUR CULTURE FROM HEART OF MOUSE 5	NORMAL RABBIT
1:10	+
1:100	+
1:1,000	+
1:10,000	−
1:100,000	−

TABLE 2—*Continued*

Passage through mouse 6

DILUTIONS OF 24 HOUR CULTURE FROM HEART OF MOUSE 6	NORMAL RABBIT
1:10	+
1:100	+
1:1,000	+
1:10,000	+
1:100,000	−

Passage through mouse 7

DILUTIONS OF 24 HOUR CULTURE FROM HEART OF MOUSE 7	NORMAL RABBIT
1:10	+
1:100	+
1:1,000	+
1:10,000	+
1:100,000	−

At this point 3 rabbits were injected. One received 1.5 cc. and another received 5 cc. of twenty-four hour culture from the heart of mouse 7. The third received the pneumococci washed from the peritoneal cavity of the same mouse. All the rabbits survived. The first and third were apparently made sick, but recovered.

The strain was then passed in rapid succession through 8 more mice. Each mouse was infected with a small drop of heart blood from the mouse dying before it. The peritoneal cavity of the last mouse was washed out with salt solution and the washings injected into a rabbit. This rabbit was somewhat younger and smaller than the former ones injected. In twenty-four hours it was dead, and a pure culture of pneumococci was recovered from its heart. The strain was passed through 2 more rabbits and recovered from the heart blood in each instance. The culture from the heart of the final rabbit was tested with the blood of 2 normal rabbits. Growth was obtained in the blood of one rabbit from a 1 : 10,000 dilution of broth culture and in the other from a 1 : 100,000 dilution (see

table 3). Before the series of passages was started this strain of pneumococci failed to grow when normal rabbit blood was seeded from a dilution of broth culture as high as 1 : 64.

This experiment shows very clearly the correlation between the virulence of pneumococci and their ability to grow in whole blood *in vitro*. The virulence of any strain of pneumococci may, therefore, be expressed with some degree of accuracy in terms of their ability to grow in blood *in vitro* as well as by the usual method of giving the number of bacteria necessary to kill an animal. Furthermore, it seems safe to assume by analogy that we may measure the virulence of a strain of pneumococci for men by measuring the ability of the pneumococci

TABLE 3

The growth of type III pneumococci in the blood of rabbits after passage through 15 mice and 3 rabbits

DILUTIONS OF 14 HOUR CULTURE FROM HEART OF RABBIT 3	NORMAL RABBITS	
	1	2
1:100	+	+
1:1,000	+	+
1:10,000	+	+
1:100,000	−	+
1:1,000,000	−	−

to grow in human blood. In doing so it must be remembered that in all probability certain men possess more or less natural immunity to pneumococci, and we must not be confused by this, because the test of the bactericidal action of whole blood records natural as well as acquired immunity.

II. THE PRODUCTION OF ANTIBODIES IN WHOLE BLOOD BY BACTERINATION

Having shown that the offensive forces of the pneumococci may be measured by blood tests *in vitro* upon normal rabbits, we attempted to determine whether or not defensive factors (antibodies) could be produced and demonstrated in the whole blood of our experimental rabbits by inoculation with pneu-

mococcus bacterins. By including, in each series of tests upon inoculated rabbits, normal rabbits as controls, it is possible to obtain assurance that the offensive forces of the pneumococci have not been materially diminished. To prevent such a diminution in the course of our experiments, the strains were occasionally passed through rabbits. Moreover, the stock strains have been carried along in rabbit blood only—artificial culture media being avoided. To determine whether the pneumococci have flourished in our capillary tubes of normal rabbit blood, one need only blow out a minute drop for microscopic examination, and the tube can be immediately resealed. If those tubes in which pneumococci have been found to grow well are placed in the refrigerator, the pneumococci will remain alive for a week, perhaps longer. When it is desirable to transplant, the contents of two or three tubes are blown out, with sterile precautions, and mixed with a few drops of sterile salt solution. A new lot of capillary tubes is seeded from this mixture and filled with normal rabbit blood, sealed, incubated and stored away. To plant a tube of broth for use on any day, a single capillary tube is opened (with precautions against contamination) and its contents blown out into the broth. Just how much this helps to prevent loss of virulence we cannot say. At least it allows the operation of natural selection among the pneumococci, because only those survive which are able to overcome whatever defensive substances the normal blood may contain.

The foremost questions that present themselves in connection with the test of the bactericidal activity of whole blood are: (1) Does the reaction appear in the blood before the agglutinins may be found in the serum, and (2) Can the test be made to serve as a quantitative measure of the extent of the immunity produced by a series of pneumococcal inoculations? These questions we have endeavored to answer.

EXPERIMENTS UPON RABBITS IN WHICH TYPE I PNEUMOCOCCI WERE USED

The strain used was obtained from The Rockefeller Institute and is known as "Neufeld."

The bacterin was prepared in the same way that Cecil and Austin (5) prepared their vaccine for use upon the division of soldiers. The pneumococci were grown for fourteen hours in infusion broth containing 1 per cent glucose. At the end of incubation the medium was heated to 53°C. for half an hour. The pneumococci were removed with the centrifuge, suspended in normal salt solution, heated to 55°C. for half hour, and 0.3 per cent tricresol added. Counts were made by Wright's methods.

Healthy, full-grown rabbits were used. They bore the inoculations without ill effects.

Tests of the bactericidal action of the blood of the rabbits were made before the inoculations were begun and at frequent intervals thereafter. Only a few of these tests need be recorded in detail.

The first series of bacterins was given subcutaneously at weekly intervals. As no bactericidal action was produced by these inoculations, only the total dosage need be set down. The first dose for each rabbit was small and it was doubled at each succeeding inoculation until four doses had been given. The results of the tests and the total dosage are combined in one table.

These same rabbits were then given intravenous inoculations of the same bacterin. The dosage and the results of the bactericidal tests are given in table 5.

The agglutinating power of the serum of these rabbits was tested before the beginning of the intravenous doses and 15 days after the final inoculation. The method of Lister (4) was used as being that best adapted to showing slight traces of agglutination. Equal volumes of serum and young broth culture were mixed and incubated in capillary glass tubes for thirty minutes. The mixtures were then blown out and care-

fully smeared over glass slides, stained and examined microscopically. Serum obtained before the first intravenous inoculation had been given was without agglutinating power. After the inoculations the serums of the two rabbits which had received the smallest dose, C and C^2 showed no agglutination although the pneumococci seemed to be less evenly distributed than in the control. The serum of rabbit C^3, which received a total of 600 millions of pneumococci agglutinated the organisms into distinct microscopic clumps. The serum of the fourth

TABLE 4

Tests of the bactericidal action of the blood of rabbits before and after subcutaneous inoculations of Type I pneumococci

DILUTIONS OF 11 HOUR CULTURE	RABBITS				
	C	C^2	C^3	C^4	N^3 (control)
Before inoculation					
1:125	+	+	+	+	+
1:625	+	+	+	+	+
1:3,125	+	+	+	+	+
Total dosage divided into 4 weekly injections	1.5 billions	3 billions	16 billions	24 billions	None
Four days after fourth inoculation					
1:125	+	+	+	+	+
1:625	+	+	+	+	+
1:3125	+	+	+	+	+

rabbit, C^4, had no effect upon the pneumococci nor had the serum of the control rabbit. Why the serum of the rabbit which received the largest doses failed to agglutinate, we do not know. We need consider only the two rabbits, C and C^2, which received the smallest doses, 60 millions and 240 millions respectively. If we grant that the slight unevenness in distribution of the pneumococci seen in the mixtures of the serum of these two rabbits with the cocci was a precursor of actual clumping, still the bactericidal power of the blood of these two rabbits was very considerable compared to that of the

control. It was so marked that it is conceivable that a smaller total dosage than 60 millions would have produced appreciable bactericidal action.

TABLE 5

Tests of bactericidal action of blood of rabbits after intravenous inoculation of type I pneumococci

	RABBITS				
	C	C^2	C^3	C^4	N^3 (Control)
First dose.....	20 millions	80 millions	200 millions	400 millions	None
6TH DAY AFTER FIRST DOSE. DILUTIONS OF 10 HOUR CULTURE					
1:25	+	+	+	+	+
1:125	+	−	+	+	+
1:625	+	+	+	−	+
1:3125	−	+	−	−	+
Second dose (8th day).....	20 millions	80 millions	200 millions	400 millions	None
16TH DAY AFTER FIRST DOSE. DILUTIONS OF 10 HOUR CULTURE					
1:25	+	+	+	−	+
1:125	−	−	+	−	+
1:625	+	−	−	−	±
1:3125	−	−	−	−	+
Third dose (16th day).....	20 millions	80 millions	200 millions	400 millions	None
25TH DAY AFTER FIRST DOSE. DILUTIONS OF 10 HOUR CULTURE					
1:25	−	−	−	+	+
1:125	+	−	−	−	+
1:625	−	−	+	−	+
1:3125	−	−	−	−	+

In our previous paper we stated briefly that the process of immunizing a rabbit against pneumococci was paralleled by an increase in the bactericidal properties of the rabbit's blood, and that the bactericidal reaction was specific to type. The

blood of a rabbit immunized with type I for instances, killed type I pneumococci but not type II or III. The experiments have since been repeated in greater detail. One revised result has been the observation that immunization to one type produces slight bactericidal action for heterogeneous types. Cecil and Austin (5) found that soldiers inoculated with types I, II, and III did not contract pneumonia due to type IV organisms. It may possibly be that inoculation with one type of pneumococci confers a slight degree of immunity of one or more other types. Agglutination does not bring this out, but the bactericidal test does.

TABLE 6

Test of the bactericidal action on type III pneumococci of the blood of rabbits after intravenous inoculation of type I

With type I pneumococci

DILUTIONS OF 10 HOUR CULTURE OF TYPE I	INOCULATED RABBITS				NORMAL RABBITS	
	C	C^2	C^3	C^4	N^3	
1:5	+	+	+	+	+	
1:25	−	−	−	+	+	
1:125	+	−	−	−	+	
1:625	−	−	+	−	+	
1:3125	−	−	−	−	+	

With type III pneumococci

DILUTIONS OF 10 HOUR CULTURE OF TYPE 3					1	2	3	4	5
1:5	+	+	+	+	+	+	+	+	+
1:25	+	−	+	+	+	+	+	+	+
1:125	+	+	−	+	+	+	+	+	+
1:625	+	+	+	+	+	+	+	+	+
1:3125	±	+	−	+	+	+	+	+	+

After the conclusion of the experiment just recorded the bloods of the rabbits C, C^2, C^3 and C^4 which possessed considerable bactericidal activity for type I, were tested on type III Pneumococci to see if any bactericidal action on this type could be detected. Five normal rabbits were used as controls. With the blood from the control rabbits growth took place in

every tube. With the blood from the rabbits inoculated with type I, Pneumococci of type III failed to grow in some 4 tubes. Experience has taught us that such irregularity is the first indication of the presence of bactericidal action, and we may therefore conclude that inoculations of one type produce slight bactericidal action for heterogenous types.

EXPERIMENTS UPON RABBITS IN WHICH TYPE II PNEUMOCOCCI WERE USED

While the experiments in which type II pneumococci were used are recorded second in this paper, they were performed sometime before those in which types I and III were used. At this time the report of Cecil and Austin's work (5) had not been published. As work done before that by these authors had shown that vaccines prepared in the usual way from pneumococci grown upon artificial culture media did not stimulate the production of demonstrable antibodies to the degree that might have been expected in view of the results of vaccination in which other bacteria had been used—*B. typhosus*, for instance —we tried to prepare a pneumoccal bacterin which should produce a better antibody response with the same or a smaller dose. A rabbit was infected intraperitoneally with a massive dose of type II pneumococci. Just before death the rabbit was chloroformed, the peritoneal cavity opened with aseptic precautions and washed out with sterile 1 per cent salt solution. This yielded 50 cc. of a suspension containing one billion pneumococci per cubic centimeter. The cells were thrown down by slow centrifugation and the supernatant fluid drawn off and heated to 53°C., for twenty minutes, phenol (0.5 per cent) being added to complete the sterilization. This vaccine was used upon one rabbit lettered "P." Lieutenant Colonel Whitmore supplied us with lipovaccine prepared from type II pneumococci in the Army Medical School, which was used upon a second rabbit lettered "L." H. K. Mulford Company supplied us with vaccine made in the usual way from several strains of type II and suspended in normal salt solution. This was used upon a third rabbit lettered "M." All three vaccines

were given subcutaneously. The culture with which the bactericidal powers of the rabbits' blood were tested came from the Rockefeller Institute and was designated D39. The dosage and the results of the bactericidal test are given in a single table.

Agglutination tests were performed upon the serum of these rabbits before the first inoculation and at frequent intervals thereafter. Lister's method was used and the technique was the same as that described in connection with type I experiments, except that 3 volumes of serum instead of 1 volume were mixed with 1 volume of broth culture. In no mixture was any indication of agglutination seen until the final test performed on the twenty-eighth day after the initial inoculation. At that time slight agglutination was produced by the serums of rabbits P and M.

On the thirty-first day after the first inoculation of bacterin these three rabbits and 2 normal control rabbits were injected intraperitoneally with 0.9 cc. of twenty-two hour blood broth culture. Our aim was to give a single minimum lethal dose. One control rabbit died in forty-eight hours and one survived; showing that the dose was a trifle below the minimum lethal dose for the strain. Rabbits L and M died in forty-eight hours and P survived.

This experiment shows that bactericidal action for type II pneumococci may be produced in the blood of rabbits as well as for type I, and that with this type also, the bactericidal test is more sensitive than the agglutination test. It also shows that a vaccine made from pneumococci grown in the living body is greatly superior in immunizing properties to one made from pneumococci grown on artificial culture media.

Because the preparation of such a vaccine can be carried out upon a small scale only, and because Cecil and Austin (5) and Lister (4) have shown that vaccine prepared from broth cultures produced satisfactory antibodies in men, we discontinued its use for the time.

Because we did not produce in rabbits satisfactory antibodies to type II pneumococci by subcutaneous inoculations of lipo-

TABLE 7

Tests of the bactericidal action of the blood of rabbits after subcutaneous inoculations of type II pneumococci

BEFORE INOCULATION DILUTIONS OF 24 HOUR CULTURE	RABBITS			
	P (peritoneal washings)	L (lipo-vaccine)	M (Salt solution suspension)	N (control)
Undiluted	+	+	+	+
1:5	+	+	+	+
1:25	+	+	+	+
1:125	+	+	+	+
1:625	+	+	+	+
First dose.....	1000 millions	1000 millions	1000 millions	None
9TH DAY AFTER FIRST DOSE DILUTIONS OF 24 HOUR CULTURE				
Undiluted	−	+	+	+
1:5	−	+	+	+
1:25	−	+	+	+
1:125	−	+	+	+
1:625	−	−	+	+
Second dose (10th day)...	2000 millions	7000 millions	2000 millions	None
Third dose (22d day).....	2400 millions	15000 millions	6000 millions	None
24TH DAY AFTER FIRST DOSE. DILUTIONS OF SALT SOLUTION SUSPENSION CONTAINING 4 BILLION COCCI PER CENTIMETER				
Undiluted	−	+	+	+
1:5	−	+	+	+
1:25	−	+	+	+
1:125	−	+	+	+
1:625	−	+	+	+
31ST DAY AFTER FIRST DOSE DILUTION OF 18 HOUR CULTURE				
Undiluted	−	+	+	+
1:5	+	+	+	+
1:25	−	+	+	+
1:125	−	+	−	+
1:625	−	+	−	−

vaccine and watery vaccine prepared in the usual way it must not be inferred that they cannot be so produced in men. The contrary has been proved many times. Upon this point Lister says (4):

> The rabbit and the man do not respond similarly to injections of the same pneumococcal vaccine. The subcutaneous administration, to a man, at appropriate intervals, of three . . . . doses . . . . of . . . . vaccine—prepared from serum glucose broth cultures—will result . . . . in satisfactory antibody production: smaller doses of the same vaccine will be equally successful if administered intravenously. A rabbit can, however, be inoculated with doses of exactly the same size of the same vaccine, either subcutaneously or intravenously, and yet no appearance of agglutinins or increase of opsonins will become apparent (page 47).

To test the comparative immunizing values of oily and watery suspensions of pneumococci some other animal than the rabbit should be used.

EXPERIMENTS UPON RABBITS IN WHICH TYPE III PNEUMOCOCCI WERE USED

The experiments in which type III pneumococci were used are the most important of the series because of the difficulty of producing a serum which will give a satisfactory agglutination of type III organisms, and because of the difficulty of producing any antibody response whatever in men by inoculations of vaccine which are well tolerated.

The Rockefeller strain, A66, was used for type III experiments. The first part of this paper records how the virulence of this strain was increased.

The experiments were arranged differently from those with types I and II. The vaccine, prepared according to the method of Cecil and Austin (5), was administered in one intravenous dose. Three rabbits were inoculated. Five days later, a fourth rabbit was given a small dose. Twelve days after the three rabbits had been inoculated and seven days after the fourth rabbit had been inoculated, all four rabbits were given,

simultaneously, 0.2 cc. of a twelve hour broth culture of living type III. Careful titration by injection into rabbits had shown this quantity to be more than 2 minimum lethal doses.

The bactericidal tests with the blood of the four rabbits, together with the results of the test dose are shown in table 8.

TABLE 8

Tests of bactericidal action of the blood of rabbits after intravenous inoculations of type III pneumococci

BEFORE INOCULATION DILUTION OF 14 HOUR CULTURE	RABBITS				
	C^5	C^6	C^7	C^3	N^4
1:5	+	+	+	+	+
1:25	+	+	+	+	+
1:125	+	+	+	+	+
1:625	+	+	+	+	+
1:3125	+	+	+	+	+
First and only dose.....	None	500 millions	1000 millions	2000 millions	None
Fifth day.....	100 millions	None	None	None	None
5TH DAY DILUTIONS OF 10 HOUR CULTURE					
1:5		+	+	+	+
1:25		+	+	−	+
1:125		+	−	−	+
1:625		−	−	−	+
1:3125		+	+	+	+
11TH DAY DILUTIONS OF 12 HOUR CULTURE					
1:5	+	+	+	+	+
1:25	−	−	+	+	+
1:125	±	−	+	−	+
1:625	+	−	−	−	+
1:3125	−	−	−	−	+
12TH DAY EACH RABBIT GIVEN 2 M. L. D. OF 12 HOUR CULTURE INTRAPERITONEALLY					
Results of test inoculation.....	Died 48 hours	Survived	Survived	Died 48 hours	Died 24 hours

In seven days the smallest dose had produced an appreciable bactericidal action in the blood of rabbit C^5, but the immunity of the animal was not sufficient to protect it against 2 minimum lethal doses. In twelve days the blood of the 3 rabbits which had received the larger doses showed distinct bactericidal action. In order to draw conclusions as to the extent of the bactericidal activity necessary to insure immunity a large series of rabbits would have to be tested.

Before the inoculations were given the agglutinating power of the sera of these rabbits was determined and the rabbits which survived the test dose, C^6 and C^7 were similarly examined three days after the dose had been given. Lister's method was again followed, one volume of serum being mixed with one volume of broth culture. No trace of agglutination was seen in any mixture.

Type III pneumococci are as susceptible to the bactericidal action of immune blood as are types I and II. As in the case of types I and II the bactericidal action appears in the blood in measurable degree before the appearance of agglutinins in the serum.

In considering this experiment as well as those in which types I and II pneumococci were used it must be remembered that our object was not to produce the maximum amount of bactericidal action, but to find out how small an inoculation of bacterin would give demonstrable bactericidal power.

DISCUSSION

The question as to the sensitiveness of the test of bactericidal action of whole blood is answered by the foregoing experiments. Those antibodies, present in blood before it coagulates, which kill pneumococci, make their appearance before those which cause agglutination of the bacteria.

Whether or not these antibodies can be measured with sufficient accuracy when the test is applied to man, is an open question which can only be answered by the performance of tests upon men similar to those we have carried out upon rab-

bits. In carrying out such experiments the most essential thing is that strains be used which have proved their ability to grow in normal human blood. It may be necessary to use freshly isolated strains.

CONCLUSIONS

1. The most general conclusion, and the most important one, to be drawn from the work upon the bactericidal activity of whole blood *in vitro* is that such blood, before it coagulates, possesses bactericidal properties which can be investigated and measured with considerable accuracy—properties which do not become apparent when blood serum alone is examined.

2. Increasing the virulence of pneumococci for rabbits increases their ability to grow in rabbit blood *in vitro*. The mathematical expression of the ability of a strain of pneumococci to grow in the blood of normal rabbits *in vitro* is an expression of the virulence of the strain for rabbits.

3. The test for the bactericidal activity on pneumococci of the whole blood of inoculated rabbits is a more sensitive test of the extent of the immunity present than is the agglutination test. This is equally true of Types I, II, and III. It is especially significant in the case of type III in which the presence of antibodies is hard to demonstrate by agglutination methods.

4. The test has an approximately quantitative value. Whether or not it can be applied successfully to the measurement of natural and artificial immunity in man can be determined only by experiment.

5. The production, by suitable inoculations, of specific bactericidal activity in the blood of rabbits, for pneumococci of one type, is accompanied by the production of slight bactericidal activity for other types.

6. A bacterin prepared from pneumococci washed from the peritoneal cavity of a rabbit dying of pneumococcal infection is more powerful as an immunizing agent for rabbits than one prepared from pneumococci grown upon artificial culture media.

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THE RÔLE OF BACILLUS INFLUENZAE IN CLINICAL INFLUENZA[1]

F. M. HUNTOON AND S. HANNUM

From the Mulford Biological Laboratories, Glenolden, Pennsylvania

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In such a complex disease as influenza, with such varied bacteriological findings, the causative factor must be a source of disputation until evidence is brought forth as to the action of the various organisms isolated.

Either one of the organisms commonly found is responsible, or a combination of two or more, or an unknown organism or virus.

Of the large number of organisms found in this condition, *B. influenzae* would appear to be the common denominator. In the hands of competent observers, employing a careful technique and an adequate menstruum this organism has been isolated from some part of the respiratory tract in a large percentage of cases. Further, organisms having the same morphological appearance have been found in microscopic sections of lungs of those dying from this disease and its complications.

The records of failure to find *B. influenzae,* which are found in the recent literature, are almost invariably unaccompanied by a statement as to the technique and medium employed or merely state that a blood agar was employed. Such results can be considered only where the conditions for the isolation and growth of the organism are known to have been favorable.

Certain observers state that after an initial failure with the employment of the usual media, success promptly followed when some of the improved media were used.

[1] Read before the New York Pathological Society March 12, 1919; before the Philadelphia Pathological Society March 27, 1919.

B. influenzae has been isolated regularly from the respiratory tract, rarely from the blood.

Since similar organisms have been isolated from a large percentage of supposed normal individuals their frequent occurrence in cases of influenza cannot be accepted alone as evidence of causation, but owing to the fact that *B. influenzae* has been isolated in pure culture from the lungs of fatal cases showing a definite pneumonia it must be considered as falling into one of the three classes; as the causative factor; as an important secondary invader, or as an unimportant secondary invader. If it falls into either of the last two classes we must look to an as yet unknown organism or virus for the initial causation.

However, if it can be shown that the *B. influenzae* or its products cause in experimental animals symptoms or lesions of the respiratory tract similar to those seen in influenza and if these effects predispose to secondary invasion of the lungs by other organisms this must be taken into account in any attempted explanation of the symptoms and pathology of the disease.

If we take into consideration the clinical picture of this disease, its pathology and the bacteriological findings, it is evident that there is a primary invasion by some organism or virus which produces a series of acute symptoms of a toxemic nature and followed in many cases by secondary invasion of the lungs by other organisms, usually of the mouth type, which, finding a fertile field, produce the various forms of pneumonia met with.

The toxemic character of the initial symptoms and the common occurrence of *B. influenzae* in the disease led us to attack the problem from the view point that this organism might, under certain conditions, be capable of producing toxic substances.

It was furthermore necessary to establish a certain amount of relationship among the various strains in order to account for the epidemiological features of the pandemic.

The finding of antibodies, agglutinins, precipitins and complement fixation bodies for *B. influenzae* in the blood of recovered cases shows merely that they have suffered from an influenzal infection, which, in a disease characterized by so many secondary invasions, may have no bearing on the primary cause, but if

antibodies from a single strain react with strains from different sources this will, at least, demonstrate a relationship between such strains and render possible an easier explanation of the epidemiological features of the disease.

Consistent negative findings in either case would militate against the consideration of this organism as a causative factor.

A report by Williams and Valentine[2] from the New York Health Department of an investigation of several strains of *B. influenzae* mostly isolated from a constricted group, apparently showed a complete lack of relationship between the various strains. This led these investigators to argue that *B. influenzae* was not the causative factor, or if it was the factor, that they had missed the pandemic strain or that the pandemic started from multiple foci of infection.

As will be seen in a later section, our results are in direct contradiction of this finding. Working with a polyvalent horse serum and monovalent rabbit and guinea-pig sera we have found a close relationship between organisms isolated in different localities, and also between these and old strains isolated in 1917 before the start of the pandemic in this country.

The relationship is shown not only by agglutinin and agglutinin absorption tests, but by protection tests against strains of virulent organisms.

The experimental evidence bearing on the problem will be divided into three sections:

1. Toxic factors in *B. influenzae* infections.
2. Serological relationship of *B. influenzae* strains.
3. The pathology of the lesions caused in experimental animals by the *B. influenzae* and its products.

Our preliminary experiments having failed to demonstrate a soluble toxin in growths of this organism, the following means were employed to extract a supposed endotoxic factor.

Ten strains of *B. influenzae* were grown in large quantities on horse blood vitamine agar, mixed with and dried *in vacuo* over P_2O_5. When perfectly dry 0.1 gram was mixed with 0.2 gram NaCl and ground for a long time in a porcelain mortar until a

[2] Read before Society of American Bacteriologists, Baltimore, 1918.

fine powder resulted. Distilled water was added drop by drop with constant stirring up to 25 cc. The salt content was, thus, approximately equal to that of physiological salt solution. This formed a heavy suspension which, when centrifuged at high speed, left a white opalescent solution, that proved to be sterile.

TABLE 1

Toxicity of salt extract (ten strains of B. influenzae dried)

EXTRACT	MICE			REMARKS
cc.				
1.0	D18*	D18	D24	
0.5	D18	D18	D18	
0.25	D24	D72	D18	Injections into the peritoneum
0.1	L	D46	D48	
0.05	L	L	D48	
0.025	L	L	L	

* In the tables presented in this article the following abbreviations are used:
D = died (the numerals indicating the number of hours that the animal lived after the injection).
L = lived.
L-s = sick; recovered.

This experiment shows a decided toxic content of the solution as prepared, but it remained to be determined whether the effect was due to a specific toxin or merely to a protein reaction.

If such a reaction is specific, one should be able to demonstrate some protection with an immune serum and no protection with normal serum.

TABLE 2

Protection tests against the toxic extract of B. influenzae with the serum from a horse immunized against the same ten strains (live organisms)

EXTRACT	SERUM	MICE		REMARKS
cc.	*cc.*			
1.0	0	D18	D24	
0.5	0	D18	D24	
0.25	0	D24	D72	
0.1	0	L	D18	Serum given simultaneously
1.0	0.1	D24	D24	
0.5	0.1	L	D36	
0.25	0.1	L	D72	
0.1	0.1	L	L	
0.5	0.5	L	L	Serum given simultaneously

These results, while showing a slight degree of protection, were not striking and a second series of tests was carried out to determine the effect of time on the combination of toxic extract and serum.

TABLE 3

Showing the effect of thirty minutes contact on the protective action of immune serum against the salt extract of B. influenzae

EXTRACT	CONTROL SET		PROTECTION TEST		
	Serum	Mice	Extract	Serum	Mice
cc.			*cc.*		
1.0	0	D24	1.0	0.1	L
0.5	0	D24	0.5	0.1	L
0.25	0	D24	0.1	0.1	L
0.1	0	D24			

The result of the tests in table 3 shows a definite inhibitory influence of the immune serum on the killing power of the extract.

TABLE 4

Comparison of the influence of immune and normal serum on the extract of B. influenzae, the sera being left in contact with the extract thirty minutes before injection

SERUM CONTROL	EXTRACT	MICE	
cc.	*cc.*		
0	0.5	D18	D18
0	0.25	D18	D24
0	0.1	D18	L
0	0.05	L	L
IMMUNE SERUM			
0.1	0.5	D18	L
0.1	0.25	L	L
0.1	0.1	L	L
NORMAL SERUM			
0.5	0.5	D18	D18
0.5	0.25	D48	D48
0.5	0.1	D18	L

The results shown in tables 2 and 3 were obtained with different lots of extract made from the same dried material. Those in table 4 with an extract from another dried lot, and show a very close correlation as to killing power.

These results also demonstrate that the toxic action of such extracts is influenced slightly when immune serum is given simultaneously and markedly when the serum and toxic extract is allowed to remain in contact for some time before injections.

They demonstrate, further, that normal serum has slight if any influence on such action.

The pathological picture presented in the animals dying from this toxic extract is remarkable. Although all the mice used were injected intraperitoneally, in no case, except when the gut had been punctured accidentally, was there any reaction visible in the peritoneal cavity but invariably the autopsy showed acutely congested lungs with areas of haemorrhage under the visceral pleura and frequently with a bloody exudate into the pleural cavity, which at times presented itself as a bloodclot.

That is, this substance has a marked action on the lungs and pleura. It was to be expected that such lesions would predispose to secondary infections of the respiratory tract and this happened at times to our experimental animals.

INFLUENCE OF SYMBIOSIS ON TOXIN PRODUCTION

An attempt was made to determine the influence of secondary infections on *B. influenzae* and on the production of toxic substances. This was carried on as follows:

A heavy emulsion of *B. influenzae* in broth was made and divided into several lots, which were treated as follows:

Lot 1. Placed at incubator temperature for forty-eight hours.

Lot 2. Inoculated with a haemolytic streptococcus and placed at incubator temperature for forty-eight hours.

Lot 3. Inoculated with the pneumococcus, type I, kept under the same conditions.

Lot 4. Same lot of broth, uninoculated, kept under the same conditions.

These lots were centrifuged then passed through Berkfeld filters and tested for toxic action with the following results:

TABLE 5

Tests of filtrates from the above emulsions

1. BROTH EMULSION B. INFLUENZAE FILTRATE	MICE	
cc.		
1.0	L	L
0.5	L	L
0.25	L	L
2. STREPTOCOCCUS-B. INFLUENZAE FILTRATE		
1.0	D48	
0.5	D48	
0.25	D48	
0.1	D72	
3. PNEUMOCOCCUS-B. INFLUENZAE FILTRATE		
1.0	L	
0.5	L	
4. BROTH CONTROL FILTRATE		
1.0	L	
0.5	L	

Table 5 shows that under the conditions of the experiment no toxin was given off by *B. influenzae* except in the presence of the growing Streptococcus hemolyticus.

To control the possibility that the streptococcus itself might be producing a toxic substance, that organism was grown for forty-eight hours in the same medium which was then filtered and tested as to its toxicity. The results of this experiment are shown in table 6.

TABLE 6

Showing the toxicity of a filtered broth culture of Streptococcus hemolyticus

STREPTOCOCCUS FILTRATE	MICE	
cc.		
1.0	D24	D48
0.5	L	L
0.25	L	L

Although a toxic substance was produced it was much weaker than the poison produced when the streptococcus was grown

in the presence of *B. influenzae*, and the pathological picture differed.

If this toxic substance produced by the symbiotic growth of the two organisms is similar in nature to the endotoxic extract it should be neutralized at least in part by the same immune serum. That this is true is shown by table 7.

TABLE 7

STREPTO-INFLUENZA FILTRATE	SERUM	MICE	
cc.	*cc.*		
1.0	0	D24	D24
0.5	0	D24	D24
0.25	0	D24	D24
0.1	0	L	L
IMMUNE SERUM			
1.0	0.2	L	L
0.5	0.2	L	L
0.25	0.2	L	L
0.1	0.2	L	L
NORMAL SERUM			
0.5	0.5	D24	D24
0.25	0.5	D48	D48
0.1	0.5	L	L

Other tests were made by combining the filtrate of streptococcus alone with the filtrate of *B. influenzae* emulsion and by adding the streptococcus filtrate to an emulsion of *B. influenzae* to determine whether a soluble ferment was responsible for the disintegration of the bacilli, but with negative results.

It may be mentioned with respect to the broth emulsion of *B. influenzae;* that when streptococcus filtrate alone is added or when *Pneumococcus* is grown in symbiosis with it, the bacilli do not disintegrate but retain both their morphology and staining characteristics, whereas in the lot grown in symbiosis with the *Streptococcus* there remain only a few organisms that could be recognized as *B. influenzae* the remainder being represented by shadow forms and detritus.

The experiments detailed above seem to be sufficient to establish that a product may be obtained from *B. influenzae* that is toxic for mice and which may be neutralized by a serum from an animal immunized to the bacilli themselves.

While this work was in progress a report was published by J. T. Parker[3] detailing work along similar lines.

In this report the author found that by growing *B. influenzae* in a veal broth to which rabbit's blood had been added, she obtained, in from eight to eighteen hours, a toxic substance which, in doses of 2 cc., killed rabbits in a few hours; she observed, also, that this substance deteriorated rapidly but that it produced immunity in recovered rabbits to subsequent fatal doses. No report was made of pathological findings nor were control series reported using uninoculated broth of the same nature.

We have repeated this work, employing the technique exactly as given and we can confirm the findings. However, we have not, as yet, obtained as powerful a toxic agent as that reported by Parker, our smallest killing dose being 4 cc. for rabbits given intravenously in one lot and 0.05 cc. for mice given intraperitoneally.

The pathological picture seen resembles much that obtained with our toxin, but without the numerous haemorrhagic foci, an acute congestion of the lungs being the most prominent lesion. Controls with the filtrate from uninoculated broth were negative.

That the broth toxin is a product of *B. influenzae* is shown by the following protection test with the serum of a horse immunized against emulsions of the live bacillus grown upon a horse blood agar.

Have these toxic substances any immunizing power?

It was found that repeated sublethal doses of the salt extract toxin and of the broth toxin immunized guinea pigs and rabbits against the homologous and heterologous toxin and also against lethal doses of the live bacilli.

Some of the characters of the two toxic substances would indicate that although somewhat similar in their action, yet they are differently constituted.

[3] Journ. A. M. A., 72, 476, 1919.

TABLE 8

Showing the neutralization of the toxic power of a broth culture of B. influenzae with immune serum

STRAIN 117 BROTH TOXIN	IMMUNE SERUM	RABBITS
cc.	*cc.*	
9	0	D2 hrs.
5	0	L
9 plus (Mixtures left at room temperature thirty minutes before injection)	1	L
	NORMAL SERUM	
8 plus (Mixture left at room temperature for thirty minutes before injection)	2	D3 hrs.

TABLE 9

Showing the neutralization of the toxic power of a broth culture of B. influenzae with immune serum

BROTH TOXIN	IMMUNE SERUM	MICE	
cc.	*cc.*		
0.9	0	D18 hrs.	D18 hrs.
0.5	0	D18	D18
0.25	0	D18	D18
0.1	0	D18	D18
0.05	0	D18	D18
0.9	0.1	L	L
0.5	0.1	L	L
0.25	0.1	L	L
0.1	0.1	L	L
0.05	0.1	L	L

Serum given simultaneously with the toxin.

The broth toxin deteriorated rapidly. Whereas 5 cc. of the freshly prepared toxin may kill a rabbit in two hours, the same material, after standing on ice for twenty-four hours, fails to kill in 10 cc. doses.

The salt extract retains its toxic power almost unchanged for at least a week.

There are differences in the appearance of the lungs from animals dead of these toxins—the salt extract producing more profound changes with a greater tendency to haemorrhage. The immune serum apparently combines with and protects against the broth toxin more readily than against the toxic agent in the salt extract (see tables 3, 4, and 9).

Although not proven, the assumption may be considered tentatively that the toxin produced in broth is of the nature of an exotoxin and that the poisonous substance contained in the salt extract is of the nature of an endotoxin.

Such a hypothesis would aid greatly in explaining the symptoms and pathology of the disease.

Are the symptoms and lesions caused by the live *B. influenzae* referable to toxic action?

The question may at least in part be answered in the affirmative. In a paper by Mr. Roos of this laboratory (see this issue) this question is taken up in detail.

It suffices here to state that animals injected with live bacilli at a site remote from the lungs, after an incubation period of several hours invariably show symptoms of respiratory distress, often with the development of nasal discharges, although at autopsy the congested lungs are bacteriologically sterile or show secondary invaders.

In two cases, however, the lungs showed a haemorrhagic pneumonia and from these *B. influenzae* was isolated.

SEROLOGICAL INVESTIGATIONS AS TO THE IDENTITY OF B. INFLUENZAE

The problem presented here was to determine the relationship of strains of *B. influenzae* from widely separated sources and to determine the possibility of the production of an immune serum of possibly curative properties.

A horse was immunized against ten selected strains, since all the information at our disposal at that time indicated that the different strains of *B. influenzae* had little or no serological relationship with one another and it was thought best to include as many of them as possible.

This serum was then tested for the presence of various antibodies.

TABLE 10

Agglutination test

HORSE SERUM	STRAIN (IMMUNIZING) B. INFLUENZAE	C	20	40	80	160	320
4271 E	140	—	4	4	4	4	4
	141	—	4	4	4	3	1
	143	—	4	4	4	—	—
	144	—	4	4	4	3	1
	139	—	4	4	4	4	4
	136	—	4	4	4	4	3
	133	—	4	4	4	4	4
	122	—	4	4	4	4	3
	117	—	4	4	4	4	4
	113	—	4	4	4	4	4

4 = Complete reaction; 3 = good reaction; 1 = trace of reaction; — = no reaction; C = control.

Complement fixation against a mixed antigen of the 10 strains showed fixation with the serum diluted 1:2000.

The results in table 10 indicate antibody formation against the various strains employed for immunization.

TABLE 11

Absorption test to determine the relationship of the strains employed for immunization and of others not so employed. Serum = Polyvalent 79699. Strain for Absorption 143 (Hygienic Laboratory 103) heavy emulsion. Dilution at which absorption occurred, 1-25. Temperature at which absorption occurred, 37°C. Absorption time, two hours

	STRAINS	C	50	100	200	400	800	1600	
Untreated serum control	117	—	1	4	4	4	3	—	Immunizing strains
	144	—	3	4	4	4	—	—	
	132	—	—	4	4	4	4	—	Not immunizing strains
	138	—	4	4	4	4	—	—	
Absorbed serum	117	—	—	—	—	—	—	—	
	144	—	3	1	—	—	—	—	
	132	—	—	—	—	—	—	—	
	138	—	—	—	—	—	—	—	

These results show; first, a distinct tendency to the pro-agglutinoid phenomenon with certain strains, a tendency that has been noted by others and secondly, an almost complete absorption of agglutinins for immunizing and heterologous strains.

TABLE 12

Absorption of monovalent rabbit sera

IMMUNIZING STRAINS	TEST STRAINS	AGGLUTINATION		
		Before absorption	After absorption	
Strain 141	141	1–320	1–10	Absorbed by strain 117
	117	1–160	1–10	
	144	1– 80	1–20	
	131	1– 20	1–10	
Strain 131	141	1–320	1–40	Absorbed by strain 144
	117	1– 20	1– 5	
	144	1–320	1–40	
	131	1–160	1–20	
	141	1–320	1–80	Absorbed by strain 117
	117	1– 20	1–10	
	144	1–320	1–40	
	131	1–160	1–10	

TABLE 13

Agglutination test with monovalent guinea-pig sera

IMMUNIZING STRAINS	TEST STRAINS	TITER
Strain 131	131	1–160
	140	1– 80
	143	1–160
	121	1–160
Strain 140	131	1–160
	140	1– 80
	143	1–160
	121	1–320
Strain 121	131	1– 80
	140	1–320
	143	1–160
	121	1–640

TABLE 14

Protection tests against various strains of B. influenzae both homologous and heterologous. Serum: polyvalent horse serum 79699. All emulsions standardized to a strength of 5000 millions to 1 cc. Injection of serum and emulsion made simultaneously into the peritoneum of white mice.

DOSE OF SERUM	DOSE EMULSION	STRAINS								
		117*	136*	141*	143*	144*	120	130	131	132
cc.	*cc.*									
0	1.0	D24	D18	D24	D18	D18	D24	D18	D18	D18
0	0.5	D18	D18	Ls.	D18	D18	D24	D18	D18	D18
0	0.25	D18	D24	D36	D24	D24	D48	L	D18	L
0	0.1	D36	D48	L	D24	L	L	L	D18	L
NORMAL										
0.5	0.5			D18	D18	D18		D18	D18	D18
IMMUNE										
0.1	1.0	L	L	L	L	L	L	D18	D48	L
0.1	0.5	L	L	L	L	L	L	L	L	D†

* Marks homologous immunizing strains.

† Death accidental.

TABLE 15

Protection against a heterologous strain 131. Serum 79699. Emulsion 10 billion to 1 cc.

IMMUNE SERUM	EMULSION	MICE	
cc.	*cc.*		
0	1.0	D24	D24
0	0.5	D24	D48
0	0.25	D24	D48
0	0.1	D24	D48
0	0.05	L	L
0.1	0.9	L	D72
0.1	0.5	L	L
0.1	0.25	L	D*

* Death accidental.

These results show a complete protection against the largest doses employed with two exceptions which are among the heterologous strains. The failure of 0.1 cc. of immune serum to protect against strain 131 completely may be accounted for by

the fact that this strain is our most virulent one and in the experiment above killed in doses of 0.1 cc. in eighteen hours. Another experiment, which will be given below, shows this same feature.

The failure to protect against strain 130 which did not kill in doses of 0.25 cc. may indicate a difference serologically. This question is taken up again at another point.

TABLE 16

Showing serological relationship of strain 130 (see table 14 and discussion). Protection test. Influenza strain 130. Serum 79699. Polyvalent. Emulsion 5000 million to 1 cc.

EMULSION	IMMUNE SERUM	MICE	
cc.	*cc.*		
1.0	0	D18	
0.5	0	D18	
0.25	0	D48	
0.1	0	L	
1.0	0.1	L	
0.5	0.1	L	
	NORMAL SERUM		
0.5	0.5	D18	D18

The results of the tests demonstrate that strains of *B. influenzae* from widely isolated sources and also strains isolated at a time when no pandemic was present have an intimate relationship and that there is nothing in the serological tests which would indicate that this organism should not be given due consideration as a causative agent.

A study of the results given in the tables above will show also that with agglutination tests alone there are differences in reaction among the different strains but that when absorption tests are employed these differences disappear.

We have found no strains among our collection which do not show relationship either directly or indirectly through absorptions.

A table giving the sources of the strains employed by us and the time of their isolation is appended.

TABLE 17

Immunizing strains for polyvalent horse serum

NUMBER	SOURCE OF STRAINS	TIME OF ISOLATION
113	Tonsilitis case	1917
117	From sputum—rhinitis case	1917
122	Tonsilitis case	1917
133	From tonsil—case of influenza	September, 1918
136	From sputum—case of influenza pneumonia	September, 1918
139	From sputum—case of influenza pneumonia	September, 1918
140	Boston strain	
141	Army strain	
143	Hygienic Laboratory strain 103	
144	Hygienic Laboratory strain 159	
	Other strains employed	
120, 130, 131, 132, 138	From tonsil; cases of influenza	September, 1918

PATHOLOGY OF EXPERIMENTAL INFECTIONS WITH B. INFLUENZAE

It was evident from the beginning of our work that our toxic products and in the case of live bacillus infections, toxic derivatives had a pronounced effect upon the smaller blood vessels with most striking results as regards the respiratory tract.

It is only possible to give a general description of the gross pathology of the lungs in these animals with a somewhat more detailed description of the microscopical picture in representative animals of each series. The latter descriptions were kindly made by Dr. Douglas Symmers to whom our thanks are due.

The results here described are divided into three groups.

1. The effects of toxic extracts of the lungs of experimental animals.

2. The lesions produced by injections of live *B. influenzae.*

3. The pathology of secondary infections, spontaneous and artificially induced.

Effects of toxic extracts of B. influenzae on the lungs of experimental animals

A. Salt extract toxin (endotoxin?). In mice, guinea-pigs and rabbits during after receiving this material, the lungs invariably showed an extreme degree of congestion, with haemorrhage in spots under the pleura and sometimes a bloody exudate in the pleural cavity.

On section the lungs showed patchy areas of haemorrhage and a marked universal congestion and they exuded a bloody froth.

The haemorrhage was frequently seen in wedge shaped areas with the base at the pleura simulating a pulmonary infarct.

Mouse. Weighing 20 grams, received 0.5 cc. of salt extract and died in eighteen hours.

Microscopical examination of the lungs showed extreme congestion of the capillaries in the interalveolar walls, with patchy areas of haemorrhage into the alveoli, associated with desquamated epithelial cells.

Pig 1. Microscopical examination of the lungs showed scattered areas of haemorrhagic extravasation and intravesicular leucocytic exudate. The intervening lung tissue was emphysematous and, in places, oedematous.

Rabbit 626. Received 5 cc. of salt extract, injected intravenously and died three hours later.

Microscopical examination of the lungs showed an acute congestion with a slight amount of intraalveolar haemorrhage and some atelectasis.

B. Broth toxin. Generally animals dead of this poison showed in the lungs an extremely acute congestion but without the extensive haemorrhage seen after injection of the salt extract.

Guinea-pig 3. Received 1 cc. of broth toxin by intraperitoneal injection and died forty-eight hours later.

Microscopical examination of the lungs showed a marked congestion with patchy areas of haemorrhage into the alveoli.

Rabbit 201. Received 10 cc. of broth toxin injected intravenously and died two hours later.

Microscopical examination of the lungs showed an extreme acute congestion.

Streptococcus—B. influenza toxin

As the pathological changes caused by this preparation were, in all respects, similar to those produced by the salt extract toxin no description of those changes will be made.

The lesions of the lungs produced by injections of live B. influenzae into the brain

This portion of the work was done in conjunction with Mr. Roos to whose paper in this issue the reader is referred for the details.

Rabbit 239. Received 2 doses of live *B. influenzae* into the brain, dying five days after the second dose.

The lungs showed acute congestion with some areas of haemorrhage.

Cultures from the lungs remained sterile.

Microscopical examination of the lungs showed a fairly intense injection of the intraalveolar capillaries with groups of atelectatic alveoli.

Rabbit 232. Received 1 dose of live *B. influenzae* into the brain, dying three days later.

The lungs showed acute congestion with haemorrhagic areas in both lower lobes. A pure culture of *B. influenzae* was obtained from both lungs.

Microscopical examination of the lung shows an intense injection of the intraalveolar capillaries together with numerous haemorrhagic extravasations into the alveoli. Some of the latter areas have fused in such fashion as to produce haemorrhages of wide extent.

Secondary infections, spontaneous and artificial

The very frequent occurrence of secondary infections in clinical influenza and the prevalence of bronchopneumonias associated with the mouth type of organisms led us to attempt the determination of the effect of the toxins of *B. influenzae* on the localization of such organisms in the lungs with their relation to bronchopneumonia.

Early in our work during an attempt to raise the virulence of a strain for mice, we had noted that four mice kept in one jar although inoculated with a pure strain of *B. influenzae* yielded on

death in cultures from the lungs a non-haemolyzing streptococcus, whereas other mice in the same series showed pure growths of *B. influenzae*.

Later two guinea-pigs which had received "influenza toxin" alone but had been left in contact with a pig receiving both the toxin and a live haemolytic streptococcus died a few days later with pneumonia, both of these animals giving pure cultures of the haemolytic streptococcus from the lungs.

In a third instance a rabbit was given a sublethal dose of toxin intravenously, and twenty-four hours later live *B. influenzae* were introduced into the nasopharynx. Death occurred in four days with both lungs yielding a pure culture of a Gram positive capsulated diplococcus, having the morphology of the pneumococcus. Sections of the lungs showed these organisms present in enormous numbers.

The above instances are sufficient to indicate that even with experimental animals suffering from this particular intoxication spontaneous secondary infections may occur with the production of pneumonic lesions.

Guinea-pig 4. Received 2 cc. of salt extract toxin by intraperitoneal injection, was kept in contact with guinea-pig 2, which had been infected with streptococcus. Guinea-pig 4 died five days after the original inoculation and three days after the death of the streptococcus pig 2.

Microscopical examination of the lung of guinea-pig 4 showed it to be the seat of a diffuse pneumonic process characterized by the presence, in the alveoli, of polynuclear leucocytes together with smaller numbers of desquamated epithelial cells, an occasional red blood corpuscle and coagulated blood serum, the whole supported in a delicate fibrinous network. The larger vessels are intensely injected. The bronchioles are filled by polynuclear leucocytes.

Rabbit 492. Received 4 cc. of extract toxin intravenously. Twenty-four hours later living *B. influenzae* were injected into the nasopharynx. The animal died four days later.

Microscopical examination of the lung showed the interalveolar capillaries to be universally injected, the small bronchioles containing numbers of polynuclear leucocytes. In patches the alveoli were filled with desquamated epithelial cells and serum. A section of the lung stained

with the Gram method showed innumerable Gram positive capsulated lance-shaped diplococci both in the alveolar walls and in the alveoli, which show the desquamation of epithelial cells.

Artificial secondary infection

Guinea-pig 2. Received 2 cc. of salt extract toxin intraperitoneally and on the next day a sublethal dose of haemolytic streptococcus by intraperitoneal injection. The animal died forty-eight hours after the injection of the streptococcus. A pure growth of *streptococcus haemolyticus* was obtained from lungs which showed marked congestion of the interalveolar capillaries with large areas of intravesicular haemorrhage and patches of leucocytic exudate into the pulmonary alveoli.

The control guinea-pig, which received the same dose of streptococcus as guinea-pig no. 2, survived.

Guinea-pig 6. Received 1 cc. of salt extract toxin intraperitoneally. One week later the animal was forced to breathe *Streptococcus haemolyticus*, dying forty-eight hours later.

Gross examination showed pneumonic consolidation of the upper lobe and portions of the lower lobe of the left lung.

Microscopical examination: The sections of the lung were unsatisfactory but they showed patchy areas in which the alveoli were filled with polynuclear leucocytes. In the intervening portions the alveoli contained fibrin with a few red cells and desquamated epithelial cells.

Sections of the lung stained with Gram's method showed the presence of innumerable streptococci.

Guinea-pig 7. Dried and ground *B. influenzae* were introduced into the naso-pharynx and one hour later the animal was forced to breathe living *B. influenzae.* Death occurred after a further twenty-four hours.

Microscopical section of the lung showed extreme congestion of the intraalveolar capillaries with a few areas of haemorrhage into the alveoli with desquamation of epithelium.

Guinea-pig 8. Received 2 cc. of salt extract material intraperitoneally and at end of forty-eight hours the animal was forced to breathe living *B. influenzae.* Death followed in twenty-four hours.

Microscopical section of the lung showed intense congestion of the intraalveolar capillaries.

Rabbits breathing toxin alone and *B. influenzae* alone, survived.

SUMMARY AND CONCLUSIONS

An examination of the experimental evidence of the preceding pages would appear to have established the following facts:

1. That *B. influenzae* is capable of producing a toxic substance.
2. That this substance, when introduced into the circulation, produces congestion of the respiratory tract with haemorrhages into the alveoli.
3. That certain conditions of symbiotic growth intensify the liberation of the toxin.
4. That as an effect of the action of the poison the lungs show a predisposition to invasion by various organisms, with the production of secondary lesions.
5. That live bacilli introduced at a remote point probably affect the lungs through the action of a liberated toxin.
6. That there is nothing in the serological evidence to preclude the consideration of this organism as an important factor in the causation of clinical influenza.

NOTES ON THE BACTERIOLOGY, AND ON THE SELECTIVE ACTION OF B. INFLUENZAE PFEIFFER

C. ROOS[1]

From the Mulford Biological Laboratories, Glenolden, Pennsylvania

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The disease commonly known as influenza has been designated in the past according to the clinical symptoms manifested in certain localities. In France it was known as *La Grippe*, from *agripper*, meaning to clutch, and is analogous to *Blitzkatarrh*, a German word, meaning lightning catarrh. Both terms are indicative of the rapid onset of the disease, which was also commonly observed during the past pandemic.

Pringle and Huxham in 1743 were the first to designate the disease by the name of influenza, from *influxus*, meaning influence of cold or influence due to atmospheric changes.

Prior to isolation and identification of *B. influenzae* by R. Pfeiffer (1,2) in 1892 the infectious rôle had been assigned to various other organisms, such as the *streptococci*, *pneumococci*, *Friedländer's bacillus*, and even the *staphylococci*, apparently as one or the other of those organisms was found to be predominant, or according to the bacteriologic technic used.

B. influenzae is an aerobic, hemophilic organism and, according to Pfeiffer, it prospers best upon pigeon blood agar, reaching a maximum growth in about twenty hours at 37°C. No growth occurs at temperatures below 28°C. nor above 42°C.

Attempts to recover *B. influenzae* from specimens twenty-four hours or more old kept at room temperature, are usually unsuccessful, even in the fall and spring months. This has been repeatedly shown in this laboratory by reculturing specimens of sputa or nasal secretions with negative results where *B. influenzae* had been found present in large numbers in the fresh specimen.

[1] Read before the Philadelphia Pathological Society, February 13, and March 27, 1919.

The usual requisite condition for the spread of the infection is close association. Since the organism is killed rapidly by exposure to low temperatures, infections through the various articles of common use or public meeting places are of less importance. There is no doubt that the spray occurring on sneezing and coughing is a potent factor in the transmission of the disease.

The bacillus is very widely distributed and frequently found present in the throats, tonsillary crypts, and larynx of apparently normal individuals producing no symptoms whatever, or it may be the cause of angina, pharyngitis, and laryngitis, without exciting the other typical symptoms of influenza. The primary lesions, however, are usually confined to the respiratory tract, most commonly the mucous membranes of the nasopharynx and nose, occasionally in the trachea and bronchi, less frequently the alveoli. (Wynekoop (3, 4), Auerbach (5), Polanski (6), Scheller (7), Jochmann (8).)

Luetscher (9), of the Johns Hopkins University, in 1915, in his studies on some 600 cases of non-tuberculous infections of the respiratory tract, found the pneumococcus and *B. influenzae* as the cause of 91 per cent of the infections of the bronchi and lungs; in these *B. influenzae* was alone in about 30 per cent in infections of the larynx, it was found in 75 per cent and in those of the nose, throat and sinuses, it was found in about 31 per cent.

The symptom complex varies greatly, apparently due to the degree of virulence of the infectious organisms, or to symbiosis with other organisms, or to the state of resistance of the individual, also to such predisposing influences as temperature and atmospheric change. (Eade (10).)

The reports as to the cause of the epidemic outbreaks of this disease as recorded by various investigators in this country and abroad have resulted generally in a repetition of inconclusive bacteriologic findings. However, it is noteworthy that the close observance of the improved bacteriologic methods has resulted in the finding of *B. influenzae* with great regularity. Indeed, the positive findings of the hemophilic bacilli of the *B. influenzae* type with improved methods of cultivation by experienced in-

vestigators have reached almost the 100 per cent level with the typical cases of the disease. As for the negative findings in the recent pandemic at least, the author is thoroughly in agreement with Park and his able coworkers that

> Those of us who have, through experience, learned of the ease with which the influenza bacillus may be missed in an examination, know how little dependence can be placed upon results of negative findings unless these reports describe fully the media used, the source and dilutions of suspected material, the time allowed for the cultures to grow and the use of specific tests and stains, and also show that all of these factors have been handled in a satisfactory way. (Keegan (12), McIntosh (13), Fildes, Baker and Thompson (14), Park, Williams and associates (11), Robertson (15), Pritchett and Stillman (22).)

An unusually high percentage of positive findings of *B. influenzae* has been reported from the various camps during the past year and also in the smaller epidemics that have occurred in this country and abroad since 1915. The pathologic lesions in general and those of the lungs in particular are claimed to be identical with those of the pandemic just past. (Christian (16), Thomas, (17), Abrahams, Hallows and French (18), Wolbach (23).)

The work in this laboratory up to the recent pandemic, includes the study of over 100 cases beginning with the epidemic of 1915–1916.

B. influenzae was found in from 50 to 90 per cent of the cases according to the time of the year and the symptoms. The presence of other organisms varied accordingly and considerably. *B. influenzae* either in pure culture or as the predominating organism, is seldom found in the specimens of nasal secretions, and then only when the symptoms are distinctly confined to the nasal cavity and characterized by copious discharge and furthermore when such specimens are collected at the very beginning of the symptoms. The author, who has been a sufferer of frequent attacks of the common cold, has been able to demonstrate this fact repeatedly on himself. Great care must be exercised to collect the proper specimen. The watery mucous discharge so abundant during the early stage of the disease contains very few

organisms of any kind. The most productive specimens are those taken from the tonsils or the posterior nasopharynx.

In a series of experiments in 1916, the author was able to confirm the observations of Jacobson (19) in regard to the symbiosis of *B. influenzae* with *streptococci* by injecting freshly isolated strains of *B. influenzae* into mice alone and in symbiosis with avirulent *streptococci*, either dead or alive. It was found that the symbiosis of these organisms increased the virulence of *B. influenzae* about ten-fold.

The observations of Cantani (20) showing the favorable influence of other organisms upon the growth of *B. influenzae* in cultures were also confirmed and have proved of considerable value in subsequent investigations.

In a series of experimental intravenous injections of rabbits with *B. influenzae* alone, and in symbiosis with streptococci and staphylococci, by making leucocytic counts ten minutes, six, twenty-four, and thirty-six hours after the injections, the following results were noted: *B. influenzae*, either alone or in symbiosis with other organisms, invariably caused a sharp drop of the leucocytes at the expense of the polymorphonuclear cells. The leucocytic count did not return to normal until about thirty-six hours after the injection. In repeated injections the same results were observed. The injection of the *streptococci* or *staphylococci* alone, on the other hand, caused a slight drop below normal shortly after injection, soon followed by sharp rise of the leucocytes, chiefly polymorphonuclear cells, to considerably above the normal count.

The blood counts during the past pandemic, as reported by numerous investigators, strikingly coincide with the above experimental results in the rabbit. (Keegan (12), Eiman (21).)

Beginning with September 19 and during the month of October, 1918, we had occasion to study 31 cases of clinical influenza, all characterized by the sharp onset of the disease. Seventeen of these cases were at the United States Naval Hospital, and 14 at the Presbyterian Hospital of Philadelphia. The sources of the cultures were as follows: throat, 10; pneumonic sputa, 12; pleural effusion (post mortem), 3; lung exudates (post mortem), 8; 33 specimens in all.

All specimens were collected with the greatest care, the throat specimens from the tonsils or posterior nasopharynx from patches showing signs of inflammation by means of sterile swabs, a tongue depressor and flash light being used. In the case of sputum the patient was asked to cough up and expectorate into a sterile receptacle. The pleural effusions and specimens of lung exudates were withdrawn by means of a sterile syringe, the chest wall being previously sterilized with tincture of iodine. These specimens were collected within an hour after death.

Most of the throat and sputum specimens were collected by the author personally, and plated within three hours after the collection on freshly made horse blood agar plates, previously tested with stock strains of *B. influenzae*. After the plates had been sown with the infectious material, in the case of swabs, by streaking directly on the plate; with the sputum, by selecting several pus kernels and washing them in sterile ascites fluid, then transferring to the blood agar plates and distributing by means of a sterile glass spreader, streaks were made of selected strains of *B. subtilis* and *staphylococci* known to influence the growth of *B. influenzae* favorably. The plates were incubated for from twenty to thirty-six hours at 37°C. and then fishings were made on blood agar slants. The delay in culturing some of the other specimens could not be avoided.

The material on the swabs, or in the case of sputum a selected portion, was suspended in sterile ascites fluid and injected into white mice intraperitoneally. These were found to die in from fourteen to thirty-six hours, except a few of those injected with the throat specimens, when the animals remained alive probably on account of the very small amount of the infectious material injected.

The mice were examined soon after death and cultures were made from the peritoneal fluid and heart blood. *B. influenzae* was recovered in most animals from the peritoneal fluid and occasionally from the heart blood as well. Other organisms recovered from the heart blood of the mice were the *pneumococci* and the *streptococci*.

The bacteriologic findings revealed the following: pneumococci

in 20 specimens: type I in 3, type II in 3, type III in 6, type IV in 8; *B. influenzae* in 27; *streptococci*, either of the hemolytic or viridans type, were found present in all of the throat and sputum specimens, absent from the three plural effusion specimens, also from 5 out of the 8 lung exudate specimens. The cultures from the throat and sputa contained a large variety of organisms besides those mentioned, such as diphtheroid bacilli, Gram negative cocci of the *M. catarrhalis* and allied types; in a number of the sputum and throat specimens a certain type of Gram positive diplococcus—an influenza like colony.

From the bacteriologic findings it will be noted that *B. influenzae* and the pneumococcus were by far the most common organisms. Streptococci as a whole were commonly present. *B. influenzae* was found in all the throat and sputum specimens and in 5 of the lung exudates, not being found in the 3 specimens of the plural effusion, in which pneumococci were present in pure culture; not present in 3 specimens of the lung exudates, from which the following organisms were isolated: *B. mucosus* and *streptococci* in one, pneumococcus type III in another; one contained no organisms, although from a specimen of sputum taken antemortem *B. influenzae* had been isolated.

With the object of establishing fixed strains of *B. influenzae* and increasing the virulence of that organism, intracranial injections of live organisms were given to rabbits, strains isolated at different localities being used as follows:

A strain from Boston, Massachusetts, a strain received from the Hygienic Laboratory, Washington, D. C.—known as the "army strain"—and a strain isolated in September, 1918, by the author from a fatal case of influenzal pneumonia at the United States Naval Hospital, Philadelphia.

The injections were made through the petrous portion of the temporal bone by means of a Record syringe and a needle such as used in the inoculation of the rabies virus by the Pasteur method. The cord was removed by the Oshida method.

The method adopted was thought superior in that use is made of comparatively well isolated organs—the brain and spinal cord, the structure and physiologic functions of which are well de-

fined; there is least danger from rapid invasion of microorganisms from other organs, and the necessary operations in regard to direct transplantation and culturing can be carried out in a sterile manner with ease. It was also considered probable that a more constant minimum lethal dose could be secured.

Tests, in dextrose bouillon fermentation tubes and blood agar plates, for purity of the organisms and freedom from external contaminations were made on all cultures and materials used for injections and were discounted from results where such were detected.

Whenever animals died within a few hours after injection, death was considered to be due to "shock."

The cultures used were from eighteen to twenty hours old, grown on blood agar, washed off in sterile ascites fluid and injected within thirty minutes to avoid autolysis as much as possible. The dose injected varied from 0.3 to 0.5 mil. The animals used were normal, healthy rabbits weighing between 1800 and 2200 grams.

The following observations were made:

The minimum lethal dose of *B. influenzae* varies considerably for the individual strains in regard to virulence, also per gram of weight for the individual animal for any one strain. There were noticeable variations of the clinical symptoms, but these were not confined to any one strain of the organisms, nor were they proportional to the amount injected per kilo of weight. The minimum lethal dose by this method was found to be about 2500 million, 3000 million, and 4000 million per rabbit of about 2000 grams, of the Boston, Army and Philadelphia strains, respectively.

It was found that the virulence of *B. influenzae* is not increased by intracranial passages through the rabbit; in fact a gradual attenuation takes place apparently on account of the insusceptibility of the rabbit to infection by this route at least. It may be noted that influenzal meningitis in human beings has been seldom observed.

Starting with a minimum lethal dose of a strain sufficient to kill the animal in about twenty hours, the spinal cord was removed immediately upon death, or the animal was chloroformed when in

the last stage. Two centimeters of the cord were cut off from the medullary end, ground up in a mortar in strictly aseptic manner, ascites fluid being used as diluent, and one-half of this amount was injected into a second rabbit. The second animal was usually found to die in from thirty-six to forty-eight hours. The third rabbit in the series injected with the same amount of material removed from the second rabbit would either survive or die from secondary infection of the lungs.

The bacteriologic examinations made of the spinal cord, lungs and heart blood, and the gross pathologic lesions observed in three separate series as above described, followed a fairly definite course. The first animal in the series showed large numbers of *B. influenzae* in the cord, the lungs and the heart blood were found sterile. Death was apparently due to acute meningitis with marked toxemia. The animal exhibiting severe meningeal symptoms with retraction of the head, appears very toxic with polypnea, a watery discharge from the nose being frequently noticed. Upon post mortem examination the lungs showed acute congestion, being filled with blood, and slightly emphyzematous. The meningeal symptoms in the second animal are less marked at first, with toxemia and polypnea, usually quite distinct in about five to six hours, but gradually though slowly increasing, up to about fifteen hours after the injection, when they may slowly recede. The bronchial symptoms, however, become more aggravated. The discharge through the nose, which at first is usually mucous and watery, becomes mucopurulent, occasionally tinged with blood pigment. Toxemia appears much increased. Breathing becomes labored but less rapid. The animal takes no food and grows weaker rapidly being unable to stand up and some times lying down for twenty-four hours before death. Post mortem examination reveals one or the other of the two more or less distinct conditions. First, little or no congestion of the brain, a considerable amount of spinal fluid, which is turbid and blood tinged. When the cord is removed *B. influenzae* is found present both in the cord and the spinal fluid in quite large numbers. The lungs are usually much congested and emphyzematous, apparently with little or no consolidation, the cultures being sterile.

Second, much congestion and emphyzema of the lungs, and very extensive consolidation and hepatization. The cultures mostly contain the Gram negative bacilli of the *B. bronchisepticus* and the *B. aerogenes* groups, streptococci, and occasionally *B. influenzae* as well. The heart blood is found sterile in either condition, the kidneys showing acute nephritis. There is no fluid in the thoracic or pericardial cavity. The third rabbit in the series shows usually only slight meningeal symptoms which soon subside. The toxemia is not very marked. The bronchial symptoms are usually more noticeable, frequently with discharge from the nose, in which case the animal is found to die from secondary infection in from three to five days. In case of death *B. influenzae* has been recovered from such animals either from the cord, heart blood, or lungs, the pathologic lesions resembling somewhat those described above.

The course of disease in one rabbit seems of special interest. The animal received supposedly a minimum lethal dose of the Philadelphia stain intracranially, showing slight meningeal symptoms and moderate toxemia in about five hours, these gradually becoming more marked but not severe. Twenty-four hours after the injection the animal was considered as recovering and seemed to be getting continually better until about forty-eight hours after the injection, when severe symptoms developed and the animal died several hours later (about sixty hours after the injection). The post mortem examination revealed the following: the brain and the spinal cord were apparently little affected (no pathologic sections were made), except a moderate amount of slightly turbid cerebro-spinal fluid, which, as well as the cord, proved sterile. The one lobe of the lungs almost entirely consolidated and having a mottled appearance, the other much congested, emphyzematous and with large hemorrhagic areas throughout. The cultural examination revealed the presence of *B. influenzae* in pure culture. The heart blood was found to be sterile. The kidneys showed acute nephritis. There was no fluid in the thoracic or pericardial cavity. Through the kindness of Dr. Huntoon, specimens of the lungs and kidney from this animal with specimens from several other animals were sub-

mitted to Dr. Douglas Symmers of the Bellevue and New York University Medical College, for the complete description of which the reader is referred to the article by Huntoon and Hannum appearing elsewhere in this issue of the Journal.[2]

Immunizations of rabbits by intracranial and intraperitoneal injections were made with the three strains individually by giving sublethal doses of the live organisms twenty hours old.

A remarkably high agglutinating titer, about 1:64, was obtained in some animals after a single intracranial injection. However, there is considerable danger of secondary bronchial infections even when a rather small initial dose is given.

For the intraperitoneal injections the proper initial dose was found to be about 3000 million, the second dose about 4000 million, and so on, at five day intervals. With an initial dose of 5000 million many animals will die after the second injection if the same dose is repeated, unless sufficient time is allowed for the animal to recover before another dose is given.

The cross agglutination and cross absorption tests with these sera against the strains used in immunization, and others isolated from this pandemic, also 6 strains isolated by the author during the epidemic of 1915–1917, showed that there is a common relationship among all of them. The slight differences noted seem to be in degree, not in kind.

Cross protection tests *in vivo* on a small number of rabbits with the intracranial method showed that although the immunity conferred was not very marked and hard to obtain, yet there is a distinct cross protection against all strains.

For more complete results pertaining to cross agglutination, cross absorption and especially cross protection, the reader is referred to the extensive and apparently conclusive experiments on the toxic substances of *B. influenzae* by Huntoon and Hannum, which have already been mentioned.

[2] Page 167.

SUMMARY AND DISCUSSION

B. influenzae can be found in every case of true clinical influenza. To isolate this organism, which is most abundant in the earlier stages of the disease, it is necessary to exercise care in obtaining a suitable specimen, and since growth requirements of this organism are quite rigid, special selective culture media, such as those suggested by Avery (24) and also by Fleming (25), carefully prepared and adjusted to reaction are essential for successful work.

The various strains of *B. influenzae* apparently do not differ in kind. This is indicated by the cross agglutination, absorption, and protection tests with strains isolated at different localities during the recent pandemic as well as with those from the epidemic of a few years ago—1915 to 1917.

The toxic substances of *B. influenzae* show a marked action on the bronchorespiratory tract, thereby predisposing these organs to extensive invasion by the organism itself or to secondary infections.

No marked increase in virulence of *B. influenzae* has been obtained by passages through laboratory animals. This, in the first place, may be due to the relative insusceptibility of these animals to the infection of this organism; secondly, to the probability that the invasive power of the organism is very limited, infection apparently taking place only when the initial toxicity is severe enough to facilitate such invasion.

No bacteremia is produced by *B. influenzae* in laboratory animals by a dose approximating a minimum lethal dose regardless of the mode of injection chosen. Rabbits receiving intracranial injections will either die of acute toxemia and show no organisms in the blood stream or lungs, or where such infection passes into the sub-acute stage there is apparently a chance for a few of the organisms to get into the blood stream and to be transferred to such organs as the lungs when these have been rendered susceptible by the toxic substances of the organism.

The pathologic lesions in the rabbit, gross and microscopic, in many respects resemble those of influenza in human beings as observed during the past pandemic.

The injection of *B. influenzae* into the rabbit intravenously results in a rapid and marked decrease in the polymorphonuclear cells.

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IMMUNOLOGIC DISPARITIES OF SPORE AND VEGETATIVE STAGES OF B. SUBTILIS

RALPH R. MELLON AND LILLIAN M. ANDERSON

From the Department of Laboratories, Hahnemann Hospital, Rochester, New York

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This study was undertaken in an effort to discover whether any necessary immunologic similarity existed between the two stages in the life history of spore bearing organisms. An organism of this type was chosen because of the unimpeachable evidence that the spore is derived from the bacillus and vice versa. We have been interested particularly in the agglutinin responses of the two stages, chiefly on account of the position taken by Eberson (1) in his study on diphtheroids.

In his attempt to discredit the contention that diphtheroids may under certain conditions assume a diplococcus morphology and, indeed, reproduce themselves as diplococci, he makes the statement that "it is obvious that, if one form is derived from another, there should be cross agglutination between the various forms." We fully admit the logic of this position, but are somewhat more interested in its truth under all conditions.

Accordingly, antispore and antibacillary sera were developed in rabbits in the following manner: A four to six hour culture of the bacilli which on staining showed no spores was killed with a 2 per cent formaldehyde solution, and controlled four days. A NaCl suspension served as the bacillary antigen. The spore antigen was prepared by growing the bacillus on plain agar containing 1 per cent CaOH for from five to ten days at 37°C. Culture killed with 2 per cent formaldehyde and controlled. Both antigens were washed three times in NaCl before injection.

PRELIMINARY AGGLUTININ REACTION

Table 1 shows the development of potent antisera, but with marked *apparent* crossing of bacillary antigen for the spore antiserum, and but a slight reaction of spore antigen with the anti-bacillary serum. It will presently be shown that in reality, no crossing occurred; for, in the case of the spore antigen particularly, all of the bacillary substance did not go into solution, and consequently, what remained, even though degenerated, still possessed agglutinogenic capacity. It is well known, of course, that

TABLE 1

	SERUM DILUTION												
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5100	1:10 M	1:20 M	1:40 M	1:80 M
Anti-bacillary serum														
Bacillary antigen	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	3+	±	0
Spore antigen	3+	2+	1+	0	0	0	0	0	0	0	0	0	0	0
Anti-spore serum. Injected spores not freed from disintegrated bacilli														
Spore antigen	4+	4+	4+	4+	4+	3+	3+	3+	2+	2+	0	0	0	0
Bacillary antigen	4+	4+	4+	4+	4+	4+	4+	0	0	0	0	0	0	0
Normal serum, control														
Spore antigen	1+	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacillary antigen	0	0	0	0	0	0	0	0	0	0	0	0	0	0

even bacterial filtrates are agglutinogenic. Zinsser (2). As could be expected, the small amount of bacillary substance mixed with the spore antigen caused a flocculation in low dilutions of the bacillary antiserum, which, however, disappeared when the latter was diluted.

The following procedure was adopted to free the spores of bacillary substance, as well as to dissolve the spore membrane, inasmuch as the density of the latter might be conceived to prevent sensitization of the contained substance. After development of spores and degeneration of the bacillary bodies, the cul-

ture was killed as usual and then treated with antiformin in concentrations of 5 and 10 per cent. With the strain used, an 18-hour exposure to 5 per cent antiformin served to dissolve the bacillary substance, as well as the membrane of part of the spores, as indicated by their inability to retain the spore stain. Of interest in this connection was the fact that, when divested of its spore membrane, the more liquid internal contents no longer presented a spherical outline, but resembled short plump bacilli, taking the counter stain. When a 10 per cent antiformin solution was employed, no acid-fast spores remained, and it is probable that many of them were completely dissolved. These results indicate that the optimum divesting concentration for the spore membrane of this strain ay between 5 and 10 per cent. Before using suspensions so treated, they were always washed three times in 0.85 NaCl solution.

TABLE 2

	SERUM DILUTION													
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5100	1:10 M	1:20 M	1:40 M	1:80 M
Anti-bacillary serum														
Five per cent antiformin spores	1+	0	0	0	0	0	0	0	0	0	0	0	0	0
Ten per cent antiformin spores	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacillary antigen	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	3+	±	0
Normal rabbit serum														
Five per cent antiformin spores	1+	0	0	0	0	0	0	0	0	0	0	0	0	0
Ten per cent antiformin spores	0	0	0	0	0	0	0	0	0	0	0	0	0	0

From the results of tables 2 and 3 it becomes increasingly evident that the apparent crossing indicated by table 1 is the result of two separate antigenic substances in the spore antigen. The spores when freed of bacillary substances show no tendency to cross agglutinate with the antibacillary serum, nor does the antispore serum cross with the bacillary antigen. In order completely

to confirm these observations, absorption reactions were carried out, as indicated in table 4.

A résumé of this procedure follows: To each of the 9 highest dilutions of the antibacillary serum, 1 cc. of a suspension of antiformin treated formalized spores was added. Separate suspensions of 5 and 10 per cent antiforminized spores were employed. After incubation for one to two hours at 37°C. they were set in the icebox overnight. In the morning they were centrifuged, the serum pipetted off and a second absorption done. After freeing the serum from the spores, the reactions indicated in the following table were performed on the same day. To be sure that no changes had taken place in the serum previously used, the observations necessary for the control of this test were repeated.

TABLE 3

Anti-spore serum

	SERUM DILUTION											
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5100	1:10 M	1:20 M	1:40 M
Ten per cent antiformin spores	4+	4+	4+	4+	4+	3+	1+	1+	0	0	0	0	0
Bacilli	0	0	0	0	0	0	0	0	0	0	0	0	0

The above table shows with finality that when the higher dilutions of an antibacillary serum are absorbed twice with either a 5 or a 10 per cent antiformin treated suspension of spores, it loses none of its agglutinins for the homologous antigen. However, the degenerated bacillary bodies (formalized spores) will absorb homologous agglutinin unless they are removed, which fact forms adequate explanation of the results in table 1. The apparent crossing can only be explained on the basis of separate antigens. The controls show that antiformin treatment does not alter the spores so as to affect their agglutinability with an homologous antiserum; neither are they spontaneously precipitated by normal serum.

These experiments are of suggestive import relative to the new antispore vaccine against anthrax, perfected by the U. S. Department of Agriculture, Bureau of Animal Industry. It is

TABLE 4

ANTIGEN	SERUM DILUTIONS													ANTISERUM
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10 M	1:20 M	1:40 M	1:80 M	
Bacilli.....						4+	4+	4+	4+	4+					Antibac. serum absorbed w. 5 per cent antiformin spores
Bacilli.....							4+	4+	4+	3+	4+	3+	2+	0	Antibac. serum w. 10 per cent antiformin spores
Bacilli.....						1	0	0	0	0	0	0	0	0	Antibac. serum abs. w. formolized spores
Formolized spores.....	1+	1+	1+	1+	1+	±	0	0	0	0	0	0	0	0	Antibac.. serum (unabsorbed)
Bacilli.....	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	3+	1+	0	Antibac. serum (unabsorbed)
Five per cent antiformin spores.....								3+	3+						Antispore serum
Five per cent antiformin spores.....	1+	0	0	0	0	0	0	0	0	0	0	0	0	0	Normal serum
Bacilli.....	+	0	0	0	0	0	0	0	0	0	0	0	0	0	Normal serum

Blank spaces in table indicate reactions were not performed.

claimed that one vaccination renders the animal immune almost immediately, making it superior to Pasteur's vaccine in every way (3).

RÉSUMÉ

1. A rabbit immunized by a culture of killed *B. subtilis* spores develops almost an equal amount of agglutinins for both spores and bacilli, unless the disorganized remnants of the latter are removed from the antigen.

2. A rabbit immunized with a culture of spore free killed bacilli will produce a potent homologous serum which shows little or no tendency to cross agglutinate in the dilutions employed; the spores do not remove the bacillary agglutinins to any extent after being treated with antiformin, but when untreated they remove them almost *in toto*.

3. Conversely, an antispore serum contains no agglutinins for the bacillary stage of the organism, and the latter does not absorb homologous antibodies from the serum.

4. Inasmuch as the bacillary agglutinins do not attack the non-acid-fast spores, their resistance to the immunological forces of the body may have an additional explanation to the one usually advanced, viz., the impenetrability of the spore wall.

5. It would seem that the agglutinin reaction is not always applicable as a means of identification of various phases of bacterial life when such exist. For, in this instance, their differences are too great to be measured by it, and it would not be surprising to find the same thing to be true of the more delicate complement fixation reaction.

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THE PERFUSION EXPERIMENT IN THE STUDY OF ANAPHYLAXIS[1]

ARTHUR F. COCA

From the Department of Bacteriology of Cornell University Medical College and the Pathological Laboratory of the New York Orthopaedic Hospital

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The question as to the site of the specific protein-antiprotein reaction that is the original cause of the symptoms of anaphylaxis may be considered settled beyond reasonable doubt. The final acceptance of the "cellular theory" of anaphylaxis has been forced by unequivocal evidence in the case of the guinea-pig as well as of the dog. A detailed analysis of this evidence is in press elsewhere (1).

One of the arguments supporting the cellular theory was gained with the use of the process of perfusion, the purpose of which was to show that a once established hypersensitiveness persists after the blood contained in the vessels has been displaced with normal blood or saline solution.

To this end the perfusions have been conducted upon the living animal, either by means of the operation of direct transfusion, as in the pioneer experiments of Manwaring (2) and in the later ones of Pearce and Eisenbrey (3), or as in the writer's (4) experiments, by the infusion of defibrinated normal blood; the same object was sought by Dale (5) and by Manwaring and Kusama (6) with the perfusion of the isolated organs of sensitized animals.

The result of all of these experiments has been the same; the characteristic reaction of anaphylaxis has always been obtainable after the perfusion, however long the process has been continued. Nevertheless, these results have not been accepted by all as

[1] This research was supported by a fund contributed by the writer's friend, Major —— ——, M.R.C., U. S. A.

evidence of the cellular site of the anaphylaxis reaction on account of the lack of proof that every trace of blood is removable from the vessels with the procedure of perfusion. This doubt has been verified, in the case of the perfusion of isolated organs, by the recent experiments of Larson and Bell (7).

These investigators have found that if the perfusion of organs is interrupted, after the last traces of blood have disappeared in the fluid issuing from the organs, corpuscles and albumin reappear in the emerging fluid when the perfusion is resumed. The explanation of this phenomenon was drawn by Larson and Bell from further experiments, in which organs were perfused with fluid containing India ink. After thorough perfusion with such fluid, only limited portions of the organs were found to be blackened by the deposit of the ink particles in the phagocytic, fixed cells of those portions. The authors' explanation is that the perfusion fluid passed through only the blackened areas, which soon become free of blood. If "the perfusion is discontinued blood from the adjacent areas of (organ) tissue diffuses into the main path of the fluid. Hence, at each renewal of the perfusion after a pause, blood and albumin reappear."

The authors conclude that the "technic (of perfusion) does not therefore remove circulating antibodies completely, as has been assumed, and this type of experiment does not establish the presence of cellular antibodies."

These conclusions seem clearly to apply to the perfusion experiment as carried out with isolated organs, especially when the conditions of the experiment demand the *complete* removal of the blood by the perfusion. However, no evidence is presented by Larson and Bell to show the applicability of their conclusions to the perfusion experiment as conducted with the living animal.

In the writer's experiments with sensitized guinea-pigs it was not necessary for his purpose to displace all of the blood in the animal's body, but only so much as to leave *less than a single minimal sensitizing quantity* and according to his calculations this requirement was amply fulfilled. In the light of the experiments of Larson and Bell, it seems necessary, now, to see whether,

in the perfusion experiment with the living animal, also, the perfusion fluid reaches only limited areas of the organs.

This investigation was conducted with the simple procedure of injecting India ink, suspended in saline solution, into the circulation of a guinea-pig. The animal was killed with chloroform and ether within two minutes after the injection and the organs were examined as to the distribution of the ink particles. The volume injected was 2 cc. The injection was made into the external jugular vein.

FIG. 1. TWO GUINEA-PIGS' LIVERS WITH THEIR RESPECTIVE CROSS-SECTIONS

The lighter one is from a normal animal; the darker one is from an animal that has received an injection of India ink.

A gross comparison was made of the organs of this animal with those of a guinea-pig that had not been injected. The lungs of the injected animal were found to be of a slightly but uniformly greyer tint than the normal lung. No distinct difference in the color of the other organs could be detected excepting the liver. This organ, as can be seen in the photograph (fig. 1), was deeply blackened and uniformly so excepting a very narrow area near the anterior border on the inferior surface.

Section of the liver showed, also, a uniform distribution of the ink particles throughout the interior of the organ.

The experiment demonstrates an immediate, complete mixing of the injected fluid with the circulating blood and it shows that if any stagnant areas exist in the lung or liver these are not extensive enough to interfere with the purpose of the perfusion.

Thus, the perfusion experiments with live sensitized animals remain as uncontroverted evidence of the cellular site of the anaphylaxis reaction.

It may be pointed out that some of the experiments of Manwaring and Kusama, although they were carried out with an isolated organ, also contribute evidence of a cellular site of reaction, inasmuch as they show a distinctly increased anaphylactic reactivity after the flushing of the vessels in the case of the "immunized" guinea-pig. This experiment demonstrates the protective action of the circulating antibodies, which points directly to a cellular reaction site. In this experiment, also, a complete removal of the blood contained in the vessels of the organ was not necessary to the purpose.

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STUDIES IN PROTEIN INTOXICATION

III. VISCERAL LESIONS IN RABBITS WITH CHRONIC PROTEIN INTOXICATION

T. HARRIS BOUGHTON

From the Department of Pathology of the University of Illinois, College of Medicine, Chicago

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Comparatively few observations are recorded on the histological changes produced by chronic or repeated protein intoxication, and these have been mostly in guinea-pigs, since rabbits are in general less well suited to this kind of work than guinea-pigs. Longcope (1) reports finding lesions consisting of necrosis, leukocytic infiltration, and fibrosis in the liver, heart, and kidney of guinea-pigs and rabbits, and in the kidneys of cats and dogs as well. The writer has previously reported (2) the finding of degenerative and necrotic lesions of the epithelium, and infiltration, fibrosis and vascular lesions in the kidneys of guinea-pigs, and also (3) vascular lesions in the smaller arteries of the liver, kidney, spleen and heart of guinea-pigs. The purpose of the present paper is to report findings in the organs of rabbits treated in a similar manner, and to point out certain differences between these and lesions of similar organs in guinea-pigs.

Forty-three rabbits were used in this work, but some died suddenly before sensitization was considered to be complete, some died from intercurrent infections, and some were found to be unsuitable for microscopic study, so that the results are based on the microscopic examination of the organs from only twenty animals. As far as possible young animals were selected so as to avoid the occurrence of spontaneous lesions. The animals were sensitized by three doses of egg-white, one subcutaneous, one intraperitoneal, and one intravenous, at intervals of from one to three days. The animals were given the first toxic in-

jection about three weeks after the last sensitizing injection. The later doses were given at varying intervals, and the size of the dose was gradually increased with the purpose of giving each animal as many anaphylactic shocks as possible, without killing the animal on the one hand, and on the other hand without producing the refractory stage. This means, of course, that many injections were not followed by a typical anaphylactic reaction. The doses varied from 0.5 cc. to 10 cc. of the albumin solution consisting of egg-white diluted with an equal volume of water. Some of the animals were under observation and being injected for periods of from seven to thirteen months, and they received as many as from 33 to 37 injections. Some animals were injected weekly, in the effort to avoid sensitization, with doses ranging from 2 cc. to 10 cc., some receiving as many as 20 to 30 doses. Several of these animals developed slight anaphylactic shocks after the third, fourth, or fifth dose, and one died of acute anaphylaxis after the third weekly dose. The animals that died of acute anaphylaxis were posted at once. Some died in the cages from several hours to a few days after the last dose, and these were posted as soon as found. The rest were killed with chloroform or ether, and posted at once. The organs were fixed in formalin, and imbedded in paraffin.

RESULTS

The most striking results were obtained in the kidneys. All of the kidneys showed pathological change. The most characteristic lesion was found in the epithelium of the convoluted tubules and in the ascending limb of Henle's loop. This change was very similar to the one previously described as found in guinea-pigs under similar conditions. It was present in all but two of the kidneys and was usually prominent. Apparently this lesion begins with a swelling or edema of the epithelial cells, which is soon followed by the appearance of vacuoles in the cytoplasm. These vacuoles increase in size until the cell presents the appearance of an irregular network, and the free margin becomes torn or frayed. This may go on to necrosis, and it

may be followed by regeneration. Invariably this change was most marked in the animals dying from acute anaphylaxis, and was less marked in the animals killed after an interval of from a few days to a few weeks after the last injection, though it was found regularly in animals killed as late as two weeks after the last injection. Almost as marked as the parenchymatous lesions was the infiltration noted. This was seen in all but one of the kidneys; it was quite distinct in most of the animals and marked in a few. In most cases the areas involved were small, but numerous, and widely disseminated over the field. In a few cases the connective tissue deposition was evidenced grossly in the dimpled appearance of the surface of the organ. In these cases the scars were quite evident microscopically as well. In more than half of the kidneys dilated tubules were found. In 20 per cent of the kidneys changes were noted in the glomeruli. This usually consisted of edema and endothelial proliferation. Some deposition of fibrin was seen, and some cicatrization. In some cases the glomeruli were increased in size. Changes were noted in the vessels in 27 per cent of the kidneys. They were slight in all cases, and consisted in a little edema beneath the internal elastic lamina of the smaller arteries.

Lesions in the hearts were almost as prominent as in the kidneys. The most striking lesion here was infiltration; this was found in every heart and it was prominent in two-thirds of the hearts. The amount of scar tissue formed, however, was not great. The areas of infiltration were small but numerous and widely distributed. Forty per cent of the hearts showed vascular lesions. These lesions were not marked, and edema of the wall was the most common change. In some cases the walls were greatly thickened and fissured. The endothelial cells were not affected. In a few instances degeneration of the cardiac muscle fibers was noted.

In the livers also the infiltration was the most prominent change. It was present in 80 per cent of the cases and it was prominent in two-thirds of the series. The amount of scar tissue formed was greater than in the hearts, but less than in the kidneys. Small areas of focal necrosis were seen in 15 per cent of the series. Vascular lesions were not seen.

Most of the spleens showed changes in the vessels. These consisted in marked thickening of the walls of the small arteries with, in some cases, fissuring of the intima. The endothelium showed no change. All of the spleens showed marked hyperemia; a few showed hemorrhage.

In the lungs the only distinct lesion was vascular. In all of the lungs it was noted that many of the small arteries had distinctly thickened walls. Of the aortas examined all were practically normal.

DISCUSSION

In general the results correspond closely to those found previously in the guinea-pigs, except that they are somewhat less severe in the rabbits. This is perhaps due to the greater difficulty with which rabbits are made anaphylactic, though it is not necessary to produce a typical shock in order to produce the lesions. Parenchymatous lesions of the kidney were less severe in the rabbits, but as frequent as in the guinea-pig. Vascular lesions were distinctly less severe in rabbits than in guinea-pigs; they were found most frequently in the kidney and not at all in the liver, whereas in the guinea-pigs they were most frequent and most severe in the liver. The occurrence of infiltration in both heart and liver was much more common in rabbits than in guinea-pigs. Areas of infiltration are occasionally found in supposedly normal rabbits, but these are much less frequent and less conspicuous than the areas found in most of the experimental animals.

Considering the duration of these experiments (in some animals from seven to thirteen months) and the size of the doses used (very frequently 5 cc. of native egg albumen) the amount of damage done is not great. This might be explained by saying that egg albumen is not very toxic to rabbits, or (what amounts to about the same thing) that the rabbit is not readily made anaphylactic. It might be pointed out that the microscopic method is not completely satisfactory for determining the amount of damage done by a certain procedure. In the animals that die of acute anaphylaxis the microscopic lesions do not seem to explain the violent symptoms or the sudden death.

CONCLUSIONS

1. Repeated injections of foreign protein in rabbits produce pathological lesions of the kidney, heart, liver, spleen and lung.

2. These lesions consist of degeneration and necrosis of the parenchymatous cells, round celled infiltration, fibrosis and edema and thickening of the walls of the smaller arteries.

3. The parenchymatous lesions were present in practically all of the kidneys, but in only a few of the hearts and livers.

4. The infiltration was present in nearly all of the kidneys, hearts, and livers and it was most prominent in the kidneys and least prominent in the hearts.

5. The vascular lesions were most frequent in the spleen, lung and heart, but they were not severe in any case.

6. In general the lesions found in rabbit organs were similar to those previously reported in guinea-pigs; the infiltration was more marked in the rabbits and the other lesions were somewhat less marked.

7. Egg albumen cannot be considered extremely toxic for rabbits, though widespread lesions are produced by the methods used.

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THE MECHANISM OF THE ANAPHYLAXIS REACTION IN THE RABBIT[1]

ARTHUR F. COCA

From the Department of Bacteriology in Cornell University Medical College and the Pathological Laboratory of the New York Orthopaedic Hospital

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The previous source of information regarding the pathology of anaphylaxis in the rabbit has been chiefly the publications of Auer. In a preliminary report (1), Auer referred the fatal acute anaphylactic shock in the rabbit to a direct effect upon the heart, which he designated as an "inhibition" or a "paralysis," and he demonstrated the local site of the anaphylaxis reaction in that animal by excluding central nervous and splanchnic influences before the reinjection. The changes in the heart described by Auer in this and later publications (2) were as follows.

> The heart is filled with blood and shows either no ventricular beat or only a weak beat. The auricles beat in some experiments slowly and regularly. The ventricles respond weakly or not at all to mechanical or electrical stimulus.
>
> There is a remarkable difference between the two ventricles; the left ventricular wall appears to be practically normal as to its consistence, whereas the right ventricular wall feels stiffer and less yielding than normal.

In a study (3) of the cause of death in rabbits resulting from the intravenous reinjection of foreign blood corpuscles, the writer came to the conclusion that the fatal result here was due to a mechanical obstruction of the pulmonary circulation by the agglutinated corpuscles. This conclusion was drawn from the fact that the corpuscles could regularly be found in the pulmo-

[1] This research was supported by a fund contributed by the writer's friend, Major ———— ————, M.R.C., U. S. A.

nany capillaries and arterioles after death resulting from the reinjection of nucleated corpuscles and from the fact that in one instance when the corpuscles were laked before they were used for the reinjection no symptoms followed that injection.

Since it seemed not impossible that the changes observed by Auer could be secondary to an interruption of the pulmonary circulation and since the histological findings in the lungs of rabbits killed by the reinjection of corpuscles had not been explained the investigation was resumed in the present study.

The first purpose of the investigation was to test directly the permeability of the pulmonary circulatory system of rabbits that had been killed with a reinjection of blood corpuscles.

Upon the advice of Dr. Robert A. Hatcher, with whom a number of the experiments were carried out, the permeability of the pulmonary vessels was tested as follows:

After the death of the animal the chest was widely opened and both ventricles of the heart were cut off with a scissors; a glass cannula, connected by rubber tubing with a glass bulb containing physiological saline solution was inserted into the pulmonary artery through the right ventricle and tied fast; care was taken not to include the pulmonary vein in the ligature; after the bulb had been placed at the level of the chest, the pinch-cock on the rubber tubing was opened and the bulb was slowly raised until fluid began to flow from the left auricle; at this point the height of the column of saline solution, measured from the level of the heart, was noted.

In two experiments on a normal rabbit and a normal cat, both killed with ether, a pressure of 10 cm. of salt solution was found to produce a steady flow from the left auricle. This result is in agreement with the estimations of the normal blood pressure in the pulmonary artery of rabbits that were reported by Knoll (4) and by Mellin (5) in two considerable series of determinations. The average pressure, according to these authors, is, repectively, 13 and 14.8 mm. of mercury. The maximal pressure recorded by Knoll was 18 mm. of mercury. In further tests with normal rabbits and guinea-pigs it was found that frequently an initial pressure of as much as 30 cm. of water was

required to inaugurate the flow of the perfusion fluid through the lungs. This was sometimes seen to be due to a twisting of the pulmonary artery just beyond the orifice of the cannula or to a closure of the orifice by the wall of the vessel being stretched over it. This circumstance obviously prevents an accurate measurement of the lesser degrees of any abnormal resistance in the pulmonary circulation.

A preliminary test of the general question under consideration was made in the following two experiments.

Rabbit 133 received a primary subcutaneous injection of 3 cc. of the once washed corpuscular sediment of oxalated sheep's blood and a second injection, 8 days later, of 2.5 cc. of the same material, this time by the intravenous route. Immediately after the death of the animal, which occurred 4 minutes after the intravenous injection, the permeability of the pulmonary vessels was tested. A pressure of 49 cm. of water caused a slight ooze of fluid from the left auricle and a pressure of 70 cm. produced a somewhat increased flow, which, however, was less than the normal flow at 10 cm.

Rabbit 303–304 received a primary intravenous injection of the washed sediment of 2 cc. of chicken's blood and, 16 days later, a similar injection of 5 cc. of the same material. Death followed the reinjection in two minutes and the pulmonary circulation was found to be impermeable to physiological saline solution under a pressure below 70 cm. of water. At 71 cm. a slight ooze of fluid from the left auricle began, which increased to a slow flow at 84 cm.

In both of these experiments, as the bulb containing the saline solution was raised in testing the permeability of the pulmonary vessels, the increasing pressure was evidenced by a ballooning of the pulmonary artery about the orifice of the cannula. When the border of the lung was incised, while the pulmonary artery was under the maximal pressure of the perfusion fluid, no oozing of fluid could be detected. A deeper incision severed the larger branches of the artery from which, then, a stream of the fluid issued freely.

These experiments demonstrate the occurrence of an obstruction to the pulmonary circulation in rabbits dying after an intravenous reinjection of blood corpuscles and they seemed

to uphold the earlier belief of the writer that the cause of that obstruction was a process of agglutination.

In support of that belief it was pointed out in the earlier publication that the serum of normal rabbits contains no agglutinins for chicken's corpuscles, which are not "toxic" on a first injection, whereas such serum does contain agglutinins for pig's corpuscles, which often kill rabbits at the first intravenous injection.

The two succeeding experiments were undertaken to determine whether death following the primary intravenous injection of "toxic" corpuscles may also be due to an obstruction of the pulmonary circulation.

Normal rabbit 408–413 received, by intravenous injection, the thrice washed corpuscles of 6 cc. of pigs blood. Death followed in four minutes and the pulmonary vessels were found to be impermeable to normal saline solution under a pressure of 90 cm. At 94 cm. a slight ooze of fluid began from the left auricle.

Normal rabbit 129–169 received a similar injection of 5 cc. of pig's corpuscles. In this instance the animal fell over two minutes after the injection but did not die till fifteen minutes later. A pressure of 29 cm. caused a slight ooze of fluid from the left auricle, which, as the bulb was raised to a height of 70 cm., increased to about the rate of flow through the normal lung at a pressure of 10 cm.

These two experiments, while apparently adding further support to the agglutination theory, actually threw some doubt upon that assumption because a previous examination had shown that the serum of rabbit 408–413 contained no demonstrable normal agglutinins for pig's corpuscles. The succeeding experiments rendered the theory untenable.

The corpuscular sediment of pig's blood, which had been washed three times with physiological saline solution, was mixed with an equal volume of distilled water and after five minutes, complete hemolysis having taken place, the mixture was centrifugalized at low speed for three minutes. The resulting supernatant fluid was injected intravenously into three normal rabbits as follows:

Rabbits 2–14 and 167–170 received respectively 8 cc. and 2.5 cc. of the supernatant fluid and both died four minutes later; the pulmonary vessels in both animals were found to be entirely impermeable to physiological saline solution under a pressure of 94 cm.; rabbit 414–415 received 8 cc. of the supernatant fluid without exhibiting any symptoms.

In only one of these three animals were normal agglutinins to pig's corpuscles demonstrable; 0.2 cc. of the serum of this animal (number 2–14) produced very slight clumping of 0.5 cc. of a 5 per cent suspension of pig's corpuscles. Normal anti-pig hemolysin was present in the sera of 2–14 and 167–170 and absent in 414–415; 0.2 cc. of the fresh active serum of the last mentioned animal caused no hemolysis of 0.5 cc. of 5 per cent suspension of pig's corpuscles.

The production of an impermeable condition of the pulmonary vessels by the injection of the corpuscular substances in solution excluded the possibility here of an agglutination phenomenon as the cause of the obstruction; the observation rendered such an explanation improbable, also, in the case of the pulmonary circulatory obstruction following the reinjection of primarily non-toxic corpuscles and it suggested that the symptoms of serum or other protein[2] anaphylaxis in the rabbit are likewise due to obstruction of the pulmonary circulation.

The mechanism of the acute shock of anaphylaxis in the rabbit was studied in 4 animals—rabbits number 405–406, 407–408, 170–304, and 409–410—which had received 13 injections of egg-white as follows: 1st day, 0.5 cc.; 6th day, 1 cc.; 8th, 9th, 10th, 11th, 13th, 14th, 15th, 16th, 17th, 18th and 20th days, 0.25 cc., usually by the intraperitoneal route. The test injections were made on the twenty-fourth day.

Rabbit 405–406 received 2.5 cc. of undiluted egg-white by intravenous injection and died two minutes later. At a pressure of 76 cm. of water a slight flow of the perfusion fluid was produced through the pulmonary vessels.

[2] A distinction between "corpuscle anaphylaxis" and "protein anaphylaxis" is made here only for convenience of discussion. It will be presently seen that, as in the guinea-pig, the mechanism of the anaphylaxis reaction in the rabbit is doubtless the same whether the anaphylactogen is formed or not.

Rabbit 407–408 was treated exactly as the preceding animal and it died within two minutes after the injection. The pulmonary vessels of this animal resisted a water pressure of 86 cm.; not the least fluid was forced through the vessels under this pressure.

The foregoing experiments demonstrate that in anaphylaxis in the rabbit the acute shock is accompanied by an obstruction to the pulmonary circulation, which, in both of the animals tested, may be considered physiologically complete.

As to the mechanism of this obstruction two possibilities suggested themselves. One of these was that a specific precipitate might be formed upon the reinjection of the protein at the test and that this precipitate could cause an embolic closure of the pulmonary capillaries and arterioles.

The possibility of a mechanical interference with the circulation by specific precipitates, formed *in vivo*, was considered in relation to the symptoms of serum allergy by Rostoski (6) and by Michaelis and Oppenheimer (7) but all of these authors rejected the theory on the ground that in such a case death must result and they concluded, from the fact that death did not follow the injection of a foreign serum into rabbits, even in the presence of demonstrable precipitin, that specific precipitates are not formed *in vivo*. The argument on which this conclusion rests is evidently invalidated in anaphylactic shock in the rabbit, in which case death does follow the injection.

In investigating the possibility under consideration the process of perfusion was resorted to with the purpose of seeing whether a removal of the greater part of the blood contained in the lungs would prevent the development of the obstruction to the pulmonary circulation when the antigen was then introduced into it.

Rabbits 170–304 and 409–410 (for previous treatment see page 222) were killed with a blow on the neck and a cannula was fastened in the pulmonary artery. With the bulb at a height of 33 cm. and 28 cm. respectively, a rapid flow of warm physiological saline solution was maintained through the pulmonary vessels for 5 minutes. The flow was then interrupted with a pinch-cock and 2.0 cc. of undiluted egg-white were injected into the lumen of the rubber tubing between the

pinch-cock and the cannula. One minute later the pinch-cock was loosened and the bulb was slowly raised. In one case (170–304) a pressure of 77 cm. of water caused a slight ooze of fluid from the left auricle; in the other (409–410) not the least fluid could be forced through the lungs under a pressure of 92 cm. of the perfusion fluid.

A certain limitation of the procedure just described has been pointed out by Larson and Bell (8), who showed that the perfusion of isolated organs with physiological saline solution does not guarantee the complete removal of the blood that is present in the organ at death, since considerable areas are not reached by the perfusion fluid. This limitation[3] cannot be disregarded. It prevents the acceptance of the foregoing results as disproving the occurrence of pulmonary embolism as a result of specific precipitate formation in the lumen of the pulmonary vessels, although these results contribute further evidence that the intravenous reinjection of an anaphylactogen in the rabbit causes a physiologically complete occlusion of the pulmonary circulation.

Further light on the question under consideration was sought with the use of toxic normal serum. It is known that in the guinea-pig and the dog, the effect, both as to symptoms and pathology, of the primary injection of toxic normal serum is indistinguishable from that of a reinjection of a non-toxic protein. This is evidently due to a peculiar susceptibility on the part of certain tissues, which are different in the two animals, (the unstriped bronchial musculature in the guinea-pig and the liver cells in the dog); a peculiar susceptibility to various agents; for example, pepton. It may be assumed, therefore, that if the injection of normal serum into the rabbit produces the symptoms and pathology of anaphylactic shock in that animal, it does so through its influence upon the mechanism that is involved in the shock of anaphylaxis.

The experiments with toxic serum (fresh unheated sheep's serum) were carried out upon two normal rabbits, x and y, of 1400 grams and

[3] It may be remarked here that while the limitation pointed out by Larson and Bell clearly applies when the perfusion is carried out on the isolated organs, it has been found (8) not to apply when the perfusion is conducted upon the living animal.

1350 grams weight, respectively. In both instances 20 cc. of the serum were injected intravenously and death occurred in $1\frac{1}{2}$ minutes and 5 minutes respectively. The pulmonary vessels in rabbit x were found to be entirely impermeable to salt solution under a pressure of 82 cm., while those of rabbit y resisted a water pressure of 68 cm. (higher pressures were not applied).

In rabbit x the right ventricle and the abdominal veins were found, at autopsy, to be completely filled with clotted blood and the possibility had to be considered that in this instance it was the clotted blood which hindered the passage of fluid through the pulmonary vessels. However, this explanation was rendered unlikely by the following experiment, which was carried out on a normal guinea-pig with the sheep's serum.

Six cubic centimeters of the serum were injected intravenously into a guinea-pig of 500 grams weight; death followed in three minutes with the typical symptoms and pathology of anaphylactic shock in this animal. The right heart was found to be filled with clotted blood, exactly as in rabbit x; yet the pulmonary vessels were readily permeable to physiological saline solution under a pressure of only 10 cm.

This experiment indicates that the mere presence of recently clotted blood in the pulmonary vessels is not sufficient to prevent the passage of fluid through them.

The results of the foregoing experiments with toxic serum indicate that the occlusion of the pulmonary vessels, which is peculiar to the pathology of anaphylactic shock in the rabbit, is not brought about by an embolic process, because the normal rabbit's blood does not possess the power of precipitating sheep's serum.

We are lead, by this conclusion, to the alternative assumption that the obstructing mechanism, which is affected both by the reaction of anaphylaxis and by primarily toxic serum, is situated in the vessel wall and that mechanism must be the muscular coat of the arteries.

It is not to be supposed that it is only the pulmonary artery of the rabbit which possesses the susceptibility that we are considering, but that this peculiarity is shared by the entire arterial

system. When the test material is administered by intravenous injection it is the pulmonary artery that comes first under its influence and it is, therefore, the interruption of the pulmonary circulation that dominates the pathology of the acute shock of anaphylaxis in the rabbit. However, when the administration is by subcutaneous injection it should be expected that a corresponding obstruction will be produced in all of the local arteries and that this will result in the formation of an area of anaemic infarction. In fact such an effect of subcutaneous injection in the rabbit has long been known in the local phenomenon of Arthus.

Thus, two of the three expressions of anaphylaxis in the rabbit are referable to the same mechanism and it remains to be seen whether the third expression of anaphylaxis; namely, that of the delayed shock or cachexia of Arthus, is susceptible of a similar explanation.

Opportunity to study the pathology of the cachexia of Arthus was presented in only one instance; hence the findings in this animal, although they were unequivocal, cannot be accepted without further confirmation. The history of the animal is as follows.

Rabbit 168 187, weighing 2800 grams, received on the 2nd, 6th and 21st of September, 2 cc., 2 cc. and 1 cc. of dog's serum injected subcutaneously. On September 28 the animal received an intravenous injection of 2 cc. of dog's serum and 2 minutes later it fell over in clonic convulsions, from which, however, it soon recovered. One further injection of 4 cc. of dog's serum was given on October 3 by the intravenous route; this injection caused only a period of drowsiness interrupted by occasional starting up. Excepting the first injection the serum used had been heated for one-half hour at 55°C.

On October 23 the rabbit was found dead and the following conditions were observed at the autopsy.

The animal was markedly emaciated; it weighed 1970 grams. The muscles were pale. The pleural cavity on both sides was filled with a clear straw colored fluid and a considerable quantity of a similar fluid was present in the abdominal cavity. The right auricle and ventricle were greatly distended and filled with a mixed red and white blood clot. The right ventricular cavity measured 1 by 1.5 cm.

The right ventricular wall presented an area of about the size of a dime which was so thin as to be translucent. This condition of the wall as well as the great dilatation of the cavity of the right ventricle is shown in the photograph (fig. 1), in which two normal rabbits' hearts have been placed on either side of the heart of anaphylactic cachexia. Microscopic examination of the thinned area revealed the entire absence of muscular elements; these were replaced with fibrous tissue. As is seen in the photograph,[4] the left ventricular cavity was relatively contracted; the left auricle was in a similar condition. The valves of the heart were normal. The lungs were diminished in volume and of markedly increased consistence. The consistence of the left lung was leathery like that of atelectasis and portions of this lung sank

FIG. 1

when placed in water. There was no edema in the lungs and no gross nor microscopic evidence of inflammation. Microscopic examination showed a thickening of the interalveolar septa, which, in the left lung, had resulted in a practically complete obliteration of the alveolar cavities. The aorta was collapsed and contained a narrow ribbon of clotted blood, which occupied but a fraction of the otherwise empty lumen. The vena cava and the abdominal veins were greatly distended with blood.

[4] In the middle of the photograph are the right and left ventricles of the heart of rabbit 168–187. The light area on the inner aspect of the right ventricle marks the area of thinning, which has been cut through. The hearts at the right and left are from normal rabbits.

All of these findings are characteristic of the effect of a chronic mitral or pulmonary stenosis, an effect which obviously must be the same if an obstruction to the circulation occurs in the lung. The assumption of the latter site of obstruction is justified here since both the mitral and the pulmonary valves were entirely normal.

In a single experiment the mechanism of acute anaphylactic shock was examined in a rabbit that had been passively sensitized.

A small normal rabbit, weighing 900 grams, received, by intravenous injection, 15 cc. of the unheated serum of a rabbit that had been highly immunized against dog's serum. Five minutes later 2 cc. of inactivated dog's serum were injected intravenously. Death followed ten minutes after this injection and the pulmonary vessels were found to be impermeable to saline solution under a pressure of less than 92 cm. of water.

We are now in position to explain the observation, reported in the earlier publication (3), that in the lung of a rabbit which has been killed by a reinjection of chicken's corpuscles the arterioles and capillaries are found to be filled with the nucleated cells and apparently occluded by them. This finding is explained by the assumption that the anaphylactogenic substances are liberated from the injected corpuscles so quickly in the blood current of the rabbit that by the time the corpuscles reach the lungs the concentration of the liberated substances is sufficient to cause the occlusive contraction of the muscular coat of the pulmonary arteries. As soon as this occlusion is physiologically complete, the flow of the blood ceases and the nucleated corpuscles remain wherever they happen to be, whether their situation is proximal or distal to the point of occlusion.

This explanation is supported by the following experiment.

Two rabbits that had received repeated intraperitoneal injections of egg-white were given finally an intravenous injection of washed chicken's corpuscles suspended in egg-white. Both animals died within a few minutes after the final injection and in both instances microscopic examination of the lungs revealed the presence of numerous nucleated corpuscles in the pulmonary arterioles and capillaries.

Excepting the experiments with nucleated corpuscles, the histological study of the lungs of normal rabbits and of those that have died in acute anaphylactic shock has failed to reveal a consistent difference in the condition of the pulmonary artery which would confirm the findings obtained with the technic described above. This study, however, will be continued.

It has been suggested above that the changes observed in the right ventricle by Auer are conceivably secondary to the interruption of the lesser circulation. Such an occurrence must affect the right heart by withholding nourishment from it and also by increasing to a maximum the work required of the right ventricle. The left ventricle is likewise deprived of nourishment, but as the systemic blood pressure falls quickly to almost zero, the left ventricle is soon relieved of the stimulus to work. This may explain the differences between the two ventricles described by Auer.

SUMMARY

In acute anaphylactic shock in the rabbit, whether induced with cells (corpuscles) or with dissolved protein, and after both passive and active sensitization, an occlusion of the pulmonary vessels is constantly observed.

Experiments with dissolved corpuscles and with primarily toxic serum indicate that this occlusion is not embolic but is due to a contraction of the muscular coat of the arteries comparable with that of the bronchial musculature in anaphylactic shock in the guinea-pig.

The local phenomenon of Arthus and the cachexia of the rabbit that was observed by the same author appear, also, to be the result of a similar interference with the circulation.

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SOME SUGGESTIVE EXPERIMENTS WITH B. INFLUENZAE; ITS TOXIN AND ANTITOXIN

A PRELIMINARY REPORT

N. S. FERRY AND E. M. HOUGHTON

From the Research Department, Parke, Davis and Company

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SUSCEPTIBILITY OF LABORATORY ANIMALS

The question as to the pathogenicity of Pfeiffer's bacillus for laboratory animals has never been given the attention it deserves, as practically all text books dismiss the subject with a very few words, leaving an impression that it is non-pathogenic, except for the human subject and perhaps the monkey.

Woolstein has called attention to the pathogenicity of this organism for the monkey, rabbit, guinea-pig and white mouse. She found that the white mouse "succumbs to intraperitoneal injections of cultures irrespective of their origin" giving rise to a peritoneal exudate containing large numbers of the influenza bacilli, as does the heart's blood. The microorganisms were found in other organs, and the spleen was always swollen.

Guinea-pigs succumb to intraperitoneal injections of one blood agar culture of all meningeal, and about one-half of the respiratory strains tested. The peritoneal fluid was increased at times to 8 cc. and the spleen was increased to two or three times its normal size. The kidneys were congested and the lungs showed scattered areas of congestion and inflammation. Influenza bacilli could be obtained in pure culture from the pleural exudate, the heart's blood and viscera and from the surface of the pia of the brain and spinal cord.

Rabbits inoculated into the ear vein succumbed in from fifteen to thirty-six hours. Small hemorrhages were found in the parietal peritoneum and within the serous coat of the intestines

and beneath the capsule of the liver, pleura and other organs. The spleen was swollen and soft, the kidneys were much congested and the lungs always showed areas of hemorrhage and of inflammation. Cultures could be obtained from the heart's blood, viscera, urine and from the surface of the brain and cord. From the congested mucous membrane of the upper nasal cavity large numbers of influenza bacilli were cultivated.

The successful production of influenzal meningitis in the monkey depended upon the selection of a virulent culture and the maintenance of the pathogenicity. This disease in the monkey terminated fatally in from thirty-six hours to four days.

The authors, working only with the rabbit, guinea-pig and white mouse, were able to corroborate the findings of Woolstein and have been able to show that the infection in these animals is a typical general infection or septicemia and that guinea-pigs and white mice were invariably susceptible to the strains at hand.

The cultures with which we have been working were obtained through the courtesy of the Hygienic Laboratory, Washington, D. C., Dr. E. C. Rosenow, of the Mayo Foundation, Rochester, Minnesota, Cook County Hospital and the New York Board of Health Laboratory, during the month of October, 1918. These cultures have all proven invariably pathogenic for guinea-pigs and mice and occasionally for the rabbit and, by means of repeated inoculations, their virulence has been increased fourfold and over. Inoculations were made intraperitoneally and intrathoracically. An invariable picture showed a general infection with intense general congestion. An intrapleural inoculation into the guinea-pig resulted in a bloody pleural effusion, usually entirely filling both pleural cavities. After peritoneal inoculations the peritoneal fluid was not, as a rule, bloody.

TOXIN PRODUCTION

Corroborating the work of Parker, we have been able to produce soluble toxins, for all strains, fatal for rabbits in intravenous doses from 2 to 5 cc.; death usually resulted in about two hours. Intense prostration within about half an hour was the

rule in all rabbits whether they died or not. In those rabbits which received nearly the fatal dose, the prostration would last several hours. The following day the rabbits gave no signs of intoxication. In producing this toxin, the technic of Parker was followed as closely as possible; the organisms were grown in veal infusion broth to which was added 10 per cent of defibrinated rabbit's blood (later on horse blood was substituted for rabbit blood). This media was prepared by heating over water bath at 75°C., or until the blood coagulated and settled on standing. In obtaining the toxin, the culture was incubated about eighteen to twenty-four hours and then centrifuged at high speed and filtered through the Mandler diatomaceous filter.

ANTITOXIN PRODUCTION

With this soluble toxic product or toxin, serum has been produced by us, in a horse, which proved bactericidal as well as antitoxic for the first set of experiments. In the production of this antitoxin the horse was injected with increasing doses of the toxin, both intravenously and subcutaneously; the injections were given every three to seven days. The first few inoculations were made with the toxin only; for the later ones the culture was centrifuged but not filtered, hence a large number of the live organisms were being inoculated into the animal at each operation. This was for the purpose of producing an antibacterial as well as an antitoxic serum. Unfortunately, before enough serum was obtained for a repetition of the experiments, the horse died; however, it was felt that the results were at least suggestive enough for a preliminary report. (Death of the horse was due to thrombo-embolic colic; in no way connected with the treatment.)

Experiments with anti-influenza serum

Experiment 1. Test of potency of anti-influenza serum from horse 902, May 22, 1919.

Serum and toxin were mixed and allowed to stand thirty minutes at room temperature.

Injections made intravenously at 9.20 a.m.

RABBIT NUMBER	SERUM	TOXIN	RESULTS
	cc.	*cc.*	
20	0.5	6	Alive
21	1.0	6	Alive
22	2.0	6	Alive
23	Control	6	Prostrated with diarrhoea at 10.05 a.m. Dead at 10.45 a.m.

Conclusion. 0.5 cc. serum protected.

Experiment II. Test of potency of anti-influenza serum from horse 902.

May 22, 1919. Serum injected intravenously fifteen minutes after toxin.

RABBIT NUMBER	SERUM	TOXIN	RESULTS
	cc.	*cc.*	
24	2	6	Dead 1 hour
25	2	6	Died 1 minute after antitoxin injection
26	Control	6	Dead 1 hour

Conclusion. No protection.

Experiment III. Test of protective value of anti-influenza serum (horse 902) against culture of *B. influenzae.* Serum injected intraperitoneally twenty-four hours before cultures, May 21, 1919. Mimimum lethal dose of culture for guinea pig, 0.25 of test tube.

GUINEA-PIG NUMBER	SERUM	CULTURE	RESULTS
	cc.		
55	1	0.75 test tube	Died May 22
56	2	0.75 test tube	Died May 22
57	3	0.75 test tube	Died May 22
58	Control	0.75 test tube	Died May 22

Conclusion. Dose of culture too large.

Experiment IV. Same test as III with smaller dose of culture.

GUINEA-PIG NUMBER	SERUM	CULTURE	RESULTS
	cc.		
59	1	0.5 test tube	Dead 5–23
60	2	0.5 test tube	Alive
61	3	0.5 test tube	Alive
62	Control	0.5 test tube	Dead 5–23

Conclusion. 2 cc. protected.

Experiment V. Test of protective value of anti-influenza serum (horse 902) against culture of *B. influenzae.*

Serum and culture injected simultaneously, intraperitoneally, May 23, 1919.

GUINEA-PIG NUMBER	SERUM	CULTURE	RESULTS
	cc.		
63	1	0.75 test tube	Dead 5–24
64	2	0.75 test tube	Dead 5–24
65	3	0.75 test tube	Alive 5–24
66	4	0.75 test tube	Alive 5–24

Conclusion. 3 cc. protected against large dose.

DISCUSSION

Irrespective of the etiological relationship of the Pfeiffer bacillus to influenza it is an interesting fact to know that a soluble toxin can be produced which will stimulate the formation of an antitoxin. It is, also, of great scientific importance to know that this antitoxin can not only neutralize the toxin *in vivo* as well as *in vitro*, but it can also protect against bacterial infection of the guinea-pig with *B. influenzae.*

Two outstanding facts should be emphasized; namely, the extreme congestion following an injection of guinea-pigs with *B. influenzae*, and the profound prostration in rabbits, due to

a toxemia, following the injection of the toxin, which very closely simulates the early stages of influenza in the human subject.

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A STUDY OF THE THERMOLABIL AND THERMOSTABIL ANTILYSINS (ANTICOMPLEMENTARY SUBSTANCES) OF HUMAN SERUM[1]

TAKAATSU KYUTOKU

From the McManes Laboratory of Experimental Pathology of the University of Pennsylvania

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That sera may develop antihemolytic properties is a well known phenomenon and particularly in connection with complement fixation tests when sera may be found to contain substances capable of interfering with hemolysis and requiring the serum control tube for its detection. The general result of a large amount of investigation on this phenomenon has been to establish the fact that these antilysins interfere with serum hemolysis by exerting some distinctive or antagonistic influence upon hemolytic complement and for this reason the phenomenon is familiarly known as the "anticomplementary" activity of serum. Furthermore, it has been shown with human sera that heating at 56°C. for thirty minutes may remove these anticomplementary substances and for this reason they are designated as "thermolabil" while in other sera the substances are not removed by this degree of heat and these are designated as "thermostabil." The sera of specimens of blood more than three days old are heated for the Wassermann test to remove the thermolabile anticomplementary substances rather than native complement which in all probability has undergone spontaneous deterioration.

Noguchi (1) gives an excellent review of the literature up to 1906 and in his studies with *dog* and *sheep* sera he found that the anticomplementary action of most sera developed after heating to 56°C. or higher due to the liberation of an antilytic substance,

[1] Presented before the annual meeting of the American Association of Immunologists, Atlantic City, June 16, 1919.

while heating to 90°C. reduced or removed this antilytic substance. Noguchi also found that his antilysin is a lipoidal substance and that it may be removed from serum by extraction with ether or, by absorption with many kinds of blood corpuscles, which thereby acquire a greater resistance to serum hemolysins. Zinsser and Johnson (2) in a study of the thermolabil anticomplementary bodies in human serum found that, unlike the thermostabil substances described by Noguchi, they could not be removed by digesting serum with red blood corpuscles and that they were allied with the globulin fraction of serum rather than the lipoidal elements. Kolmer (3) in studies concerning the phenomenon of non-specific complement fixation by normal *rabbit, dog* and *mule sera* found that the anticomplementary activity was increased by heating at 56°C. for thirty minutes followed by a decrease when heated at 62°C. and entire removal by heating at 70°C. for thirty minutes. Blood corpuscles were found to absorb a portion of these antilytic substances. Both the serum lipoids and proteins (particularly the globulins) were found to be concerned in the antilytic and non-specific complement fixation reactions with normal rabbit and dog sera (4).

PURPOSES OF INVESTIGATION

Inasmuch as Noguchi and Kolmer worked with dog, rabbit, ox and sheep sera while Zinsser and Johnson employed human sera, the differences in the results may have been due to the fact that the phenomenon varies according to the sera of different animals; for example, human sera when heated apparently do not develop the anticomplementary properties found by Noguchi and Kolmer with rabbit, dog, mule, ox and sheep sera. At the suggestion of Professor Kolmer, I have undertaken to study the anticomplimentary substances in human sera after the following plan, in order to determine whether more light may be thrown upon the mechanism of this interesting phenomenon and a means discovered for its removal, in view of the very practical bearing that these problems have upon diagnostic complement fixation tests.

1. The influence of heating human sera in relation to anticomplementary substances.

2. The relation of bacteria to the development of anticomplimentary substances in serum.

3. The anticomplimentary activity of hemoglobin in human sera.

4. Changes in reaction and hydrogen ion concentration of anticomplimentary human sera.

5. The relation of the proteins of human serum to the phenomenon of anticomplimentary activity, as determined by refractometric studies.

6. The relation of ether soluble lipoids to the phenomenon of anticomplimentary activity of human serum.

7. Experiments bearing upon the removal of anticomplimentary substances from human serum by methods of absorption and filtration.

Preliminary experiments have shown that the antilytic substances in serum act upon complement, which is in entire accord with the numerous investigations of others; in my experiments these substances were found to have no direct antilytic effect upon corpuscles alone nor hemolysin alone, but have shown a direct antagonistic or neutralizing effect upon complement. My experiments also support the statements of Zinsser and Johnson that in all probability these antilytic substances do not ordinarily exist preformed as such in sera, although they may be occasionally encountered in perfectly fresh sera, but rather they are secondary products of development in sera under certain conditions.

GENERAL TECHNIC

The majority of the sera used in this study were secured from syphilitic individuals undergoing treatment in the clinic of Dr. Schamberg, and were employed fresh and unheated and after being heated at 56°C. for thirty minutes. Throughout this paper unless otherwise stated, "heated" refers to this degree and duration of heating in a water bath.

Tests for anticomplimentary activity were ordinarily conducted by placing in a series of chemically clean and sterile test tubes increasing amounts of serum with a constant amount of complement fixed at 1 cc. of a 1:20 dilution of the sera of guinea-pigs and sufficient physiological salt solution to make the total volume about 2 cc. These mixtures were then incubated in a water bath at 38°C. for one hour and the degree of anticomplementary influence determined by adding two units of hemolysin and 1 cc. of a 2.5 per cent suspension of washed sheep cells to each tube followed by reincubation in a water bath for one hour and readings after the tubes had stood in the refrigerator at 0 to 2°C. overnight.

The hemolysin was always titrated with each complement serum and corpuscle suspension for accurate adjustment in the hemolytic system.

The usual hemolytic system and corpuscle controls were included in each experiment.

PART ONE

The influence of heating human sera in relation to anticomplementary substances

While heating the sera of the dog, rabbit, ox, and sheep at temperatures between 50 and 60°C., may increase the antilytic activity as shown by Noguchi and Kolmer, similar changes do not occur with human sera.

As shown in table 1, unheated human sera may be anticomplementary while heating at 56°C. tends to remove these thermolabile anticomplementary substances. This experiment was conducted with ten sera collected without special precautions and kept for five days at room temperature. All were anticomplementary before heating while after heating at 56°C. for thirty minutes none were anticomplementary, showing the influence of heat in removing these antilytic substances. Additional experiments conducted with sera heated for thirty minutes at 60°C., 70°C., 80°C. and 90°C. have shown that antilysins do not develop as described by Noguchi with the sera of the lower animals.

Thermostabil anticomplementary substances resist heating at 50°C. for as long as two hours as shown in table 2 in an experiment conducted with four sera; likewise these heat resisting

TABLE 1

The influence of heat on serum containing thermolabil anti-complimentary substances

SERUM NUMBER	AMOUNT OF SERUM	UNHEATED	5 MINUTES*	10 MINUTES	15 MINUTES	20 MINUTES	25 MINUTES	30 MINUTES
	cc.							
1	0.2	M.H.†	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
2	0.2	N.H.	S.H.	M.H.	C.H.	C.H.	C.H.	C.H.
3	0.2	N.H.	N.H.	N.H.	N.H.	S.H.	M.H.	C.H.
4	0.2	N.H.	N.H.	N.H.	S.H.	C.H.	C.H.	C.H.
5	0.2	N.H.	N.H.	V.M.H.	C.H.	C.H.	C.H.	C.H.
6	0.2	N.H.	N.H.	N.H.	S.H.	M.H.	C.H.	C.H.
7	0.2	N.H.	N.H.	N.H.	V.S.H.	M.H.	M.H.	C.H.
8	0.2	M.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
9	0.2	N.H.	S.H.	M H	M.H.	M.H.	C.H.	C.H.
10	0.2	N.H.	S.H.	M.H.	C.H.	C.H.	C.H.	C.H.

* Min. = Minute.

† C.H. = Complete hemolysis; M.H. = Marked hemolysis; S.H. = Slight hemolysis; N.H. = No hemolysis.

TABLE 2

The influence of heat on sera containing thermostabil anti-complimentary substance

SERUM NUMBER	AMOUNT OF SERUM	UNHEATED	5 MINUTES	10 MINUTES	15 MINUTES	20 MINUTES
	cc.					
1	0.2	N.H.	S.H	S H	M H.	M.H.
2	0.2	S.H.	M.H.	M.H.	V.M.H.	V.M.H.
3	0.2	N.H.	S.H.	M.H.	M.H.	M.H.
4	0.2	S.H.	M.H.	V.M.H.	V.M.H.	V.M.H.

SERUM NUMBER	AMOUNT OF SERUM	25 MINUTES	30 MINUTES	45 MINUTES	60 MINUTES	90 MINUTES	120 MINUTES
	cc.						
1	0.2	M.H.	M.H.	M.H.	M.H.	M.H.	M.H.
2	0.2	V.M.H.	V.M.H.	V.M.H.	V.M.H.	V.M.H.	V.M.H.
3	0.2	M.H.	V.M.H.	V.M.H.	V.M.H.	V.M.H.	V.M.H.
4	0.2	V.M.H.	V.M.H.	V.M.H.	V.M.H.	V.M.H.	V.M.H.

antilytic substances may not be entirely removed by heating at 60°C. for thirty minutes as shown in table 3 in an experiment with twelve sera.

As shown in table 1 thermolabil anticomplementary substances may be removed by heating at 56°C. for five to thirty minutes; the difference in time required for their removal is partly quantitative inasmuch as sera containing most antilytic substances before heating require the longer exposures. Temperatures below 50°C. appear to have but slight influence upon these anticomplementary substances while a rapid reduction occurs at temperatures between 50° and 60°C.

TABLE 3

The influence of heat on sera containing thermostabil anti-complementary substances

SERUM NUMBER	AMOUNT OF SERUM	UNHEATED	40°C.*	45°C.	50°C.	55°C.	60°C.
	cc.						
1	0.2	N.H.	N.H.	N.H.	N.H.	N.H.	V.S.H.
2	0.2	N.H.	N.H.	N.H.	N.H.	N.H.	V.S.H.
3	0.2	N.H.	N.H.	N.H.	N.H.	S.H.	S.H.
4	0.2	N.H.	N.H.	N.H.	N.H.	S.H.	M.H.
5	0.2	N.H.	N.H	N.H.	N.H.	N.H.	N.H.
6	0.2	N.H.	N.H.	N.H.	N.H.	V.S.H.	V.S.H.
7	0.2	N.H.	N.H.	N.H.	N.H.	S.H.	M.H.
8	0.2	N.H.	N.H.	N.H.	N.H.	S.H.	S.H.
9	0.2	V.S.H.	V.S.H.	V.S.H.	S.H.	S.H.	S.H.
10	0.2	V.S.H.	V.S.H.	V.S.H.	V.S.H.	M.H.	C.H.
11	0.2	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.
12	0.2	N.H.	N.H.	N.H.	S.H	M.H.	M.H.

* In the water bath for thirty minutes.

PART TWO

The relation of bacteria to the development of anticomplementary substances

1. Sterile broth and egg albumin do not develop anticomplementary substances. These experiments were conducted in order to determine whether plain neutral broth containing the usual amounts of protein in the form of pepton and beef extract and

egg white, develop anticomplementary properties when kept under sterile conditions in an incubator at 37°C. This temperature was chosen as being most favorable for the occurrence of chemical changes.

The results of an experiment with steril broth neutral in reaction to phenolphthalein and titrated for anticomplementary activity after incubation from one to twenty-five days, are shown in table 4, and indicate that while broth in large doses may contain thermolabil and thermostabil anticomplementary substances in mixture with 1 cc. of 1:20 pig serum, there is no increase in

TABLE 4

The anti-complementary activity of plain steril neutral broth

AMOUNT OF BROTH (UNDILUTED)	1 DAY		5 DAYS		10 DAYS		25 DAYS	
	Unheated*	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.								
0.05	C.H.*	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.2	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.3	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.5	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H	C.H.
1.0	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
1.5	V.M.H.	V.M.H.	V.M.H.	C.H.	V.M.H.	C.H.	C.H	C.H.
2.0	M.H.	M.H.	M.H.	C.H.	V.M.H.	C.H.	C.H.	C.H.
3.0	N.H.	N.H.	S.H.	M.H.	M.H.	V.M.H.	V.M.H.	M.H.
Control	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

* C.H. = complete hemolysis; V.M.H. = very marked hemolysis; M.H. = marked hemolysis; S. H. = slight hemolysis; N.H. = no hemolysis.

this antilytic activity over a period of twenty-five days. As a general rule, heating broth at 56°C. for thirty minutes reduced the antilytic activity.

The results of an experiment with steril egg albumin shown in table 5, indicates that this substance rich in proteins, does not become anticomplementary under steril conditions.

2. Steril human sera develop thermolabil anticomplementary substances. In the majority of instances fresh steril human sera are not anticomplementary as tested with a satisfactory complement of the sera of guinea-pigs; as shown in these experiments,

however, steril human sera develop thermolabil but not thermostabil anticomplementary substances when kept sealed in ampules over a period of time. As shown in tables 6, 7, and 8 steril sera develop thermolabil anticomplementary substances in from three to seven days when kept hermetically sealed in ampules at 37°C.

TABLE 5

The anti-complimentary activity of egg albumin

AMOUNT OF EGG WHITE (1; 5)	RESULTS	
	First day	After 7 days
cc.		
0.2	C.H.*	C.H.
0.4	C.H.	C.H.
0.6	C.H.	C.H
0.8	C.H.	C.H.
1.0	C.H.	C.H.
2.0	C.H.	C.H.
Control	C.H.	C.H.

* C.H. = complete hemolysis.

TABLE 6

The anti-complementary activity of steril sera kept in an incubator

AMOUNT OF SERUM (1:10)	FIRST DAY		AFTER 4 DAYS		AFTER 7 DAYS		AFTER 17 DAYS	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.								
0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.5	C.H.	C.H.	C H.	C.H.	C.H.	C.H.	C.H.	C.H.
1.0	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
1.5	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	M.H.	C.H.
2.0	C.H.	C.H.	C.H.	C.H.	V.M.H.	C.H.	M.H.	C.H.
3.0	C.H.	C.H.	V.M.H.	C.H.	M.H.	C.H.	S.H.	C.H.
Control	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

but thermostabil substances do not develop during periods as long as seventeen days.

At room temperature thermolabil substances develop in about seven days (table 9) but in the refrigerator (temperature 0–2°C.) these did not develop over a period of thirty-one days (table 10). Thermostabil anticomplementary substances did not develop in any specimen.

TABLE 7

The anti-complementary activity of steril sera kept in an incubator

AMOUNT OF SERUM (1:10)	FIRST DAY		AFTER 4 DAYS		AFTER 7 DAYS		AFTER 17 DAYS	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.								
0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.5	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	V.M.H.	C.H.
1.0	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	M.H.	C.H.
1.5	C.H.	C.H.	C.H.	C.H.	V.M.H.	C.H.	M.H.	C.H.
2.0	C.H.	C.H.	C.H.	C.H.	V.M.H.	C.H.	M.H.	C.H.
3.0	C.H.	C.H.	C.H.	C.H.	M.H.	C.H.	M.H.	C.H.
Control	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

TABLE 8

The anti-complementary activity of steril sera kept in an incubator

AMOUNT OF SERUM (1:10)	FIRST DAY		AFTER 3 DAYS		AFTER 7 DAYS		AFTER 10 DAYS	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.								
0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.5	C.H.	C.H.	C.H.	C.H.	V.M.H.	C.H.	C.H.	C.H.
1.0	C.H.	C.H.	V.M.H.	C.H.	V.S.H.	C.H.	S.H.	C.H.
1.5	C.H.	C.H.	S.H.	C.H.	V.S.H.	C.H.	S.H.	C.H.
2.0	C.H.	C.H.	S.H.	C.H.	N.H.	C.H.	N.H.	C.H.
3.0	C.H.	C.H.	V.S.H.	C.H.	N.H.	C.H.	N.H.	C.H.
Control	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

TABLE 9

The anti-complementary activity of human sera kept at room temperature

AMOUNT OF SERUM	1 DAY		7 DAYS		31 DAYS	
	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.						
0.01	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.05	C.H.	C.H.	C.H.	C.H.	S.H.	C.H.
0.1	C.H.	C.H.	C.H.	C.H.	S.H.	C.H.
0.2	C.H.	C.H.	V.M.H.	C.H.	N.H.	C.H.
0.3	C.H.	C.H.	V.M.H.	C.H.	N.H.	C.H.
Control	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

TABLE 10

The anti-complementary activity of steril sera kept in a refrigerator (0 to 2° C.)

AMOUNT OF SERUM	1 DAY		7 DAYS		31 DAYS	
	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.						
0.01	C.H.	C.H.	C.H.	C.H.	S.H.	C.H.
0.05	C.H.	C.H.	C.H.	C.H.	N.H.	C.H.
0.1	C.H.	C.H.	C.H.	C.H.	N.H.	C.H.
0.2	C.H.	C.H.	C.H.	C.H.	N.H.	C.H.
0.3	C.H.	C.H.	C.H.	C H	N H.	C.H.
Control	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

3. Serum dried in filter paper does not become anticomplementary. Ordinary fresh human serum collected under usual conditions, with no special aseptic technic and containing bacteria upon culture, was quickly dried by fanning, in Schleich and Schull's paper no. 597 and kept in tightly stoppered bottles at room temperature. Anticomplementary tests with these fresh sera before and after heating and in amounts up to 0.3 cc. showed the complete absence of anticomplementary substances.

Subsequent tests with the dried paper at intervals up to twenty days as shown in table 11 indicated thermolabil and thermostabil anticomplementary substances do not develop under these conditions.

4. Sera containing bacteria develop thermolabil and thermostabil anticomplementary substances. In striking contrast to these results with steril sera are the results of experiments with sera contaminated with various microorganisms and particularly staphylococci; contaminated sera invariably develop thermolabil and thermostabil anticomplementary properties as described by Craig (5).

In table 12 are shown the results of anticomplementary tests with plain neutral broth kept in an incubator for three days; one portion was kept steril and a second portion was inoculated with *Staphylococcus albus*. Titrations show that the latter became highly anticomplementary and particularly when used unheated.

TABLE 11

The anti-complementary activity of serum dried on filter paper

SIZE OF DRIED PAPER	CORRESPONDING AMOUNT OF SERUM (1:10)	1 DAY OLD				5 DAYS OLD				10 DAYS OLD				20 DAYS OLD			
		Active serum		Inactive serum		Active serum		Inactive serum		Active serum		Inactive serum		Active serum		Inactive serum	
		Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
mm.	*cc.*																
5 x 10	0.15	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
5 x 20	0.3	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
5 x 40	0.6	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
5 x 60	0.9	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
5 x 80	1.2	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
5 x 100	1.5	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
5 x 150	2.25	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
5 x 200	3.0	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
Control	0	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

TABLE 12

The anti-complementary activity of steril and staphylococcus broth

AMOUNT OF BROTH (UNDILUTED)	STERIL BROTH		STAPHYLOCOCCUS BROTH	
	Unheated	Heated	Unheated	Heated
cc.				
0.1	C.H.	C.H.	C.H.	C.H.
0.2	C.H.	C.H.	C.H.	C.H.
0.4	C.H.	C.H.	V.M.H.	C.H.
0.6	C.H.	C.H.	V.M.H.	C.H.
0.8	C.H.	C.H.	M.H.	C.H.
1.0	C.H.	C.H.	S.H.	C.H.
2.0	C.H.	C.H.	S.H.	V.M.H.
Control	C.H.	C.H.	C.H.	C.H.

In table 13 are shown results of tests with the two sterile human sera kept at room temperature sealed in ampules for a period of two weeks; a portion of each serum was inoculated with *Staphylococcus albus*. As shown in this table the steril portions of each serum developed thermolabil anticomplementary properties as previously described while those portions containing staphylococci developed more of this variety of anticomplementary substance and thermostabil antilytic substances in addition.

TABLE 13

Anti-complementary activity of steril human serum and serum containing staphylococci *

AMOUNT OF SERUM (1:10)	SERUM I				SERUM II			
	Steril		Staphylococci inoculated serum		Steril		Staphylococci inoculated serum	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.								
0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	S.H.	C.H.
0.5	S.H.	C.H.	C.H.	C.H.	M.H.	C.H.	N.H.	V.M.H.
1.0	V.S.H.	C.H.	M.H.	C.H.	N.H.	C.H.	N.H.	M.H.
1.5	N.H.	C.H.	S.H.	C.H.	N.H.	C.H.	N.H.	S.H.
2.0	N.H.	C.H.	S.H.	C.H.	N.H.	C.H.	N.H.	N.H.
3.0	N.H.	C.H.	N.H.	V.M.H.	N.H.	C.H.	N.H.	N.H.
Control	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

* These steril and staphylococcus inoculated sera were kept at room temperature for two weeks.

While staphylococci occur most frequently among contaminated microorganisms found in sera as ordinarily collected, and produce thermostabil and thermolabil antilytic substances, other microorganisms are capable of producing the same changes. In table 14 are shown the results of an experiment conducted by dividing a steril human serum into five parts and inoculating four parts with *Staphylococcus albus*, *B. coli*, *B. typhosus* and *B. subtilis* respectively reserving the fifth part as steril serum. All were sealed in ampules and placed in an incubator for ten days when anticomplementary tests were conducted with each portion

TABLE 14

*The rôle of various bacteria in producing anti-complementary substances in human sera**

AMOUNT OF SERUM (1:10)	STERIL		STAPHYLOCOCCUS		B. TYPHOSUS		B. COLI		B. SUBTILIS	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.										
0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.5	C.H.	C.H.	V.M.H.	V.M.H.	C.H.	C.H.	C.H.	C.H.	V.M.H.	C.H.
1.0	C.H.	C.H.	N.H.	N.H.	V.M.H.	C.H.	S.H.	V.M.H.	S.H.	V.M.H
2.0	C.H.	C.H.	N.H.	N.H.	S.H.	C.H.	N.H.	S.H.	N.H.	V.M.H.
3.0	C.H.	C.H.	N.H.	N.H.	N.H.	C.H.	N.H.	S.H.	N.H.	S.H.
Control	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

* Each serum was inoculated with an equal amount of culture of each germ.

before and after heating at 56°C. for thirty minutes. As shown in this table the steril portion did not become anticomplementary whereas the four portions containing the bacteria developed thermolabil and thermostabil anticomplementary substances and especially those contaminated with staphylococci and *B. subtilis*.

A question of considerable interest in this connection is whether the cocci themselves or their products act as antilytic substances. To answer this question an experiment was conducted by dividing a steril rabbit serum into three parts as follows: One part was sealed in ampules and kept steril; a second portion was inoculated with staphylococci and incubated for five days. At the end of this time the number of cocci per cubic centimeter was counted

and an equal number of washed cocci from agar cultures were added to the third portion of steril serum carried over in sealed ampules in an incubator. The results of anticomplementary tests upon the three portions before and after heating are shown in table 15; the steril portion had not become anticomplementary whereas both portions containing staphylococci developed thermolabil and thermostabil anticomplementary substances. Inasmuch as the addition of washed cocci to sterile serum rendered the latter anticomplementary it is logical to conclude that

TABLE 15

The anti-complementary activity of steril and contaminated rabbit sera

AMOUNT OF SERUM (1:10)	B SERUM*		C SERUM		D SERUM	
	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.						
0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.2	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.4	C.H.	C.H.	C.H.	C.H.	C.H	C H
0.6	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.8	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
1.0	C.H.	C.H.	M.H.	C.H.	C.H.	C.H.
2.0	C.H.	C.H.	N.H.	M.H.	V.M.H.	V.M.H.
3.0	C.H.	C.H.	N.H.	S.H.	N.H.	M.H.
Control	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

*b = steril serum kept in an incubator for five days; c = serum inoculated with staphylococci and kept in an incubator for five days; d = steril serum to which washed staphylococci were added.

the cocci themselves irrespective of their products, are antilytic, which is in accord with the well known anticomplementary activity of bacterial-antigens in general.

Similar results were observed in an experiment in which plain steril neutral broth was employed. One portion was placed in an incubator for five days; a second portion was inoculated with *Staphylococcus albus* and the number of cocci per cubic centimeter was counted at the end of five days. To the third portion, carried over in the incubator for five days, was added a corresponding number of washed cocci from agar cultures and anticomplementary tests were conducted with the three portions

before and after heating. As shown in table 16 the steril portion remained free of antilysins whereas both portions containing cocci developed thermolabil and thermostabil anticomplementary substances.

As will be described later, the removal of these cocci from serum and broth by porcelain filtration removed the anticomplementary activities of both.

TABLE 16

The anti-complementary activity of steril and contaminated broth

AMOUNT OF BROTH (UNDILUTED)	B BROTH*		C BROTH		D BROTH	
	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.						
0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.2	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.4	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.6	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.8	C.H.	C.H.	V.M.H.	C.H.	C.H.	C.H.
1.0	C.H.	C.H.	V.M.H.	C.H.	M.H.	V.M.H.
2.0	C.H.	C.H.	M.H.	M.H.	N.H.	N.H.
3.0	C.H.	C.H.	M.H.	S.H.	N.H.	N.H.
Control	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

*b = steril broth kept in an incubator for five days; c = broth inoculated with staphylococci and kept in an incubator for five days; d = steril broth to which washed staphylococci had been added.

PART THREE

The anticomplementary activity of hemoglobin in human serum

Since the sera of old specimens of blood collected under usual conditions, deeply tinged with hemoglobin and usually contaminated with bacteria, frequently prove anticomplementary both before and after heating, experiments have been conducted for the purpose of determining whether the products of disintegration of erythrocytes alone may contribute the antilytic substances.

A solution of fresh sheep cells washed four times was prepared in 100 cc. of steril distilled water by dissolving as much blood as possible with gentle shaking; this solution was filtered through paper and rendered isotonic with sodium chlorid. Anticomple-

mentary tests conducted with the fresh solution before and after heating showed the material to be highly anticomplementary inasmuch as amounts as low as 0.1 cc. contained sufficient thermolabil and thermostabil anticomplementary substances completely to inhibit hemolysis as shown in table 17.

Additional experiments were conducted by collecting human blood under sterile conditions into test tubes and conducting tests with a portion of the sera for anticomplementary activity before and after heating. In one experiment the remainder of the serum was left on the clot in an incubator for eight days,

TABLE 17

The titration of anti-complementary activity of sheepcell hemoglobin

AMOUNT OF HEMOGLOBIN SOLUTION (UNDILUTED)	FIRST DAY		AFTER 10 DAYS	
	Unheated	Heated	Unheated	Heated
cc.				
0.1	N.H.	N.H.	N.H.	N.H.
0.2	N.H.	N.H.	N.H.	N.H.
0.4	N.H.	N.H.	N.H.	N.H.
0.6	N.H.	N.H.	N.H.	N.H.
0.8	N.H.	N.H.	N.H.	N.H.
1.0	N.H.	N.H.	N.H.	N.H.
2.0	N.H.	N.H.	N.H.	N.H.
Control	C.H.	C.H.	C.H.	C.H.

at which time it was discolored a deep red with liberated hemoglobin but steril when cultured; anticomplementary tests with this serum before and after heating showed that it was highly antilytic, especially before heating (table 18).

In a second experiment conducted in the same manner with two sera but kept at room temperature and titrated for anticomplementary activity at intervals, showed the development of thermolabil anticomplementary substances in about ten days, at which time the sera contained from 20 to 25 per cent of hemoglobin (table 19).

TABLE 18

Anti-complementary properties of hemoglobin in steril serum

AMOUNT OF SERUM (1:10)	FIRST DAY		AFTER 8 DAYS*	
	Unheated	Heated	Unheated	Heated
cc.				
0.1	C.H.	C.H.	C.H.	C.H
0.5	C.H.	C.H.	M.H.	C.H
1.0	C.H.	C.H.	S.H.	C.H.
1.5	C.H.	C.H.	S.H.	M.H.
2.0	C.H.	C.H.	N.H.	S.H.
3.0	C.H.	C.H.	N.H.	S.H.
Control	C.H.	C.H.	C.H.	C.H.

* This serum was steril upon culture.

TABLE 19

The anti-complementary activity of hemoglobin in steril serum

AMOUNT OF SERUM (UNDILUTED)	1 DAY				4 DAYS			
	No. 1		No. 2		No. 1		No. 2	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.								
0.05	C.H.	C.H.	C.H.	C.H.	C.H.	C H	C.H.	C.H.
0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.2	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.3	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
Control	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

AMOUNT OF SERUM (UNDILUTED)	7 DAYS				10 DAYS			
	No. 1		No. 2		No. 1*		No. 2	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.								
0.05	C.H.	C.H.	C.H	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.2	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.3	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	V.M.H.	C.H.
Control	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

* Serum 1 was steril upon culture at end of ten days; but serum 2 was contaminated. These sera developed a deep red color at the end of seven to ten days.

PART FOUR

Changes in reaction of anticomplementary sera

In view of the fact that minute traces of inorganic acids and alkalies have been found highly anticomplementary, (6) experiments have been conducted to determine whether changes in the reaction of steril and contaminated sera may be detected by colorimetric methods as these sera develop thermolabil and thermostabil antilysins.

In studying changes in the reaction of the sera a new indicator was employed described by Bronfenbrenner (7) and composed of equal parts of a one-half per cent watery solution of China blue with a 1 per cent solution of rosolic acid in 95 per cent alcohol designated as C-R. In preliminary experiments I have found that this indicator may be prepared free of bacteria and as tested with dilutions of normal sodium hydroxid and normal hydrochloric acid is at least four to six times more delicate an indicator than phenolphthalein; anticomplementary titrations of serum alone and serum plus ten drops of indicator per cubic centimeter have shown no increase of antilytic activity of the latter.

The addition of one drop of C-R to each cubic centimeter of fresh, clear and almost colorless human serum imparts a purplish red color indicating neutrality; incubation of clear steril serum plus the indicator sealed in ampules over a period of three weeks, usually caused a slightly deeper red color, indicating a change of reaction in the direction of alkalinity and titrations at this time showed the presence of small amounts of thermolabil anticomplimentary substances.

Similar experiments conducted with almost colorless sera contaminated with *Staphylococcus albus* showed that production of alkaline changes as indicated by the development of redder tints over a period of ten days, after which no further changes in color could be detected; at the end of twenty days these sera were highly anticomplementary before and after heating.

Studies bearing upon changes in the hydrogen ion concentration of steril and contaminated sera during the time anticomple-

mentary substances were being formed, were attempted by means of a colorimetric method. The results of these experiments indicate that no definite information was to be obtained by this method. Specimens of human sera were collected under rigid aseptic conditions and in glassware prepared with particular care to insure chemical cleanliness. Anticomplementary tests and hydrogen ion determinations were conducted with each of the fresh sera, following which each specimen was divided into two portions and treated as follows: the first portion was kept steril and the second inoculated with *Staphylococcus albus*, both being placed in prepared ampules hermetically sealed in an incubator for seven days. Fresh steril serum showed a Ph of 7.6 to 7.8 and was free of anticomplementary activity before and after heating; after seven days in an incubator steril sera showed a Ph of 7.8 to 8.0 and thermolabil anticomplementary substances, while the contaminated sera showed a Ph of 7.4 to 7.6 and both thermolabil and thermostabil antilysins.

PART FIVE

Refractometric studies of anticomplementary sera

As previously stated, Zinsser and Johnson have found that the thermolabil anticomplementary substances of human serum are removed by precipitating the globulins with ammonium sulphate; Kolmer found a reduction in the antilytic properties of dog, rabbit and mule sera with similar methods. These investigations indicate therefore that the anticomplementary substances of serum are, at least in part, associated with the protein constituents and especially the globulins.

In this investigation studies were conducted after the refractometric method of Robertson (8); the results of a series of experiments are shown in table 20.

In conducting these experiments steril and contaminated human sera were employed; anticomplimentary and refractometric tests were conducted with each serum while fresh and then repeated seven to twenty days later; the sera were kept in an incubator sealed in ampules to prevent evaporation.

TABLE 20

Protein changes in anti-complementary sera as determined by the refractometric method

SERA NUMBER	CONDITION OF SERA	ANTI-COMPLEMENTARY TESTS*		RESULTS OF REFRACTOMETRIC DETERMINATIONS			
		Thermolabile	Thermostabile	Total protein	Albumin	Globulin	Non-protein
		cc.	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*
1	Fresh	0†	0	8.3	5.6	2.7	1.1
	7 days; contaminated	0.05	0.05	9.8	9.3	0.5	1.6
2	Fresh	0	0	8.7	5.6	3.1	1.2
	7 days; contaminated	0.05	0.05	10.0	7.9	2.1	1.1
3	Fresh	0	0	7.7	3.7	4.0	1.6
	20 days; contaminated	0.05	0.3	10.2	9.2	1.0	1.9
4	Fresh	0	0	7.9	4.8	3.1	1.4
	20 days; contaminated	0.05	0.05	11.2	10.3	0.9	1.8
5	Fresh	0	0	8.7	7.5	1.2	1.6
	20 days; steril	0.15	0	9.0	6.0	3.0	1.8
6	Fresh	0	0	7.2	5.7	2.5	1.4
	20 days; steril	0.15	0	9.0	4.9	4.1	1.8
7	Fresh	0	0	7.2	6.2	1.0	1.6
	7 days; steril	0.1	0	8.9	6.4	2.5	1.0
	7 days; contaminated	0.05	0.05	8.6	6.6	2.0	1.0
8	Fresh	0	0	8.5	7.7	0.8	1.8
	7 days; steril	0.1	0	8.5	5.5	3.0	1.3
	7 days; contaminated	0.05	0.05	9.7	7.8	1.9	1.3
9	Not filtered	0.1	0.3	8.7	6.6	2.1	1.3
	Filtered‡	0	0	4.2	4.0	0.2	1.3
10	Not filtered	0.05	0	7.6	6.1	1.5	2.0
	Filtered	0	0	3.4	3.2	0.2	2.0
11	Unheated	0.06		8.0	7.7	0.3	3.3
	Heated§		0.2	8.2	7.4	0.8	2.4
12	Unheated	0.02		8.6	7.0	1.6	2.4
	Heated		0.2	8.1	6.8	1.3	2.3

* Smallest amounts of undiluted serum proving anti-complementary are given in this table.

† 0 = Not anti-complementary in dose 0.3 cc. undiluted serum which was the maximum dose tested.

‡ Filtered through Kitasato candle filter.

§ Heated in a water bath at 56°C. for thirty minutes.

Owing to the individual variations in the amounts of albumin and globulin in the different sera, the results of these studies will not bear too close analysis, but a general analysis warrants the following conclusions:

1. Contaminating sera proving highly anticomplementary before and after heating have shown an increase of the total protein and especially of the albumin fraction; it is reasonable to infer that these changes are due to the presence of bacteria.

2. Sterile sera containing thermolabil anticomplementary substances showed a slight increase of total protein and especially of the globulin fraction.

3. Sera strongly anticomplementary before and after heating and filtered through Kitasato filters, which removed both the thermolabil and thermostabil antilysins, showed a decrease in total proteins and especially of the globulin fraction.

4. Refractometric determinations of anticomplementary sera before and after heating showed practically no changes in the protein constituents even though there was a marked reduction but not a complete removal, of the anticomplementary substances.

PART SIX

The relation of ether soluble lipoids to the anticomplementary activity of human serum

As previously mentioned, Noguchi has found that the antilysins liberated in dog, ox, and sheep serum as a result of heating at 50 to 60°C. may be removed by heating the serum with ether and the extract, freed from lecithin and certain related bodies, contains the antilysin in a concentrated but not in a pure form, which can now be taken up in a saline solution in which it dissolves. Kolmer found that the dog and rabbit sera extracted with ether were more antilytic than untreated portions of the same sera until they were heated at 56°C. when the extracted portions were less antilytic; that is, extraction of those sera with ether increased the thermolabil anticomplementary activity but reduced the thermostabil substances.

In my experiments conducted with anticomplementary human sera, extraction with ether did not remove the thermolabil or

thermostabil anticomplementary substances, but rather brought about such changes as to increase the antilytic activity of the sera. In these experiments sera were titrated for antilytic activity before and after heating; 3 cc. of each were then extracted with 10 cc. of ether and the latter was carefully separated in the centrifuge and decanted into an evaporating dish. The residue complementary tests were then repeated with the sera before and

TABLE 21

The extraction of anticomplementary sera with ether

NO.	SERA	ANTICOMPLEMENTARY TITRATIONS					
		Unheated serum			Heated serum		
		0.05	0.1	0.2	0.05	0.1	0.2
1	Plain serum	N.H.*	N.H.	N.H.	M.H.	C.H.	C.H.
	Plain serum after extraction	S.H.	N.H.	N.H.	M.H.	S.H.	S.H.
	Ether residue in saline	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
2	Plain serum	N.H.	N.H.	N.H.	C.H.	C.H.	C.H.
	Plain serum after extraction	N.H.	N.H.	N.H.	S.H.	N.H.	N.H.
	Ether residue in saline	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
3	Plain serum	N.H.	N.H.	N.H.	S.H.	N.H.	N.H.
	Plain serum after extraction	M.H.	S.H.	N.H.	S.H.	S.H.	N.H.
	Ether residue in saline	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

*N.H. = No hemolysis (strongly anticomplementary); S.H. = slight hemolysis; M.H. = marked hemolysis; C.H. = complete hemolysis.

of serum was then extracted once more with the same volume of ether and the latter was decanted into the same dish. Anti- after heating and with the same complement serum; the ether was evaporated and the residue was taken up in 3 cc. of physiological saline solution and tested for anticomplementary activity before and after heating in the same manner as the sera. The results of an experiment with three sera are shown in table 21;

sera 1 contained large amounts of thermolabil and small amounts of thermostabil antilytic substances; serum 2 contained thermolabil antilysins only while serum 3 contained large amounts of both.

As shown in this experiment extraction with ether did not remove the thermolabil antilysins except to a slight extent in serum 3, and saline solutions of the ethereal residues did not contain antilytic substances; as a general rule extraction with ether increased the amounts of thermostabil antilysins inasmuch as the antilytic activity of all sera heated after extraction with ether was higher than that with the plain sera after heating.

PART SEVEN

The removal of anticomplementary substances from human serum by methods of absorption and filtrations

1. The influence of absorption with barium sulphate. Wechselmann and Lange have shown that absorption of syphilitic sera with barium sulphate increases the delicacy of the Wassermann test probably by removal of natural antisheep hemolysin as shown by Noguchi and Bronfenbrenner; experiments have been conducted with anticomplementary sera to determine if barium sulphate, kaolin, bone ash and other substances were capable of removing the antilysins.

In one set of experiments sera were diluted 1:10 and titrated for antilytic activity before and after heating and portions of 2 cc. were treated with 1.1 cc., 5.5 cc. and 11 cc. of a 7 per cent suspension of barium sulphate in physiological saline solution; these mixtures were incubated for an hour and the barium was removed by centrifugation. The supernatant sera were collected and further diluted with saline solution until the final dilution was 1:10 when the antilytic tests were repeated before and after heating; the results are shown in tables 22 and 23.

As shown in these tables barium sulphate is capable of absorbing a portion of the antilysins of human sera and especially thermolabil antilysins; the larger amounts of barium removed more antilysin than the smaller amounts.

TABLE 22

The absorption of thermostabil and thermolabil antilysins by barium sulphate

AMOUNT OF SERUM (1:10)	UNTREATED SERUM		NO. 1*		NO. 2		NO. 3	
	Unheated	Heated.	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.								
0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.2	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.3	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.4	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.5	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.6	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.7	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.8	V.M.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.9	V.M.H.	V.M.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
1.0	M.H.	V.M.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
2.0	N.H.	N.H.	S.H.	M.N.	V.M.H.	V.M.H.	S.H.	S.H.
3.0	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.

* No. 1 = 2 cc. serum + 1.1 cc. of 7 per cent $BaSO_4$; No. 2 = 2 cc. serum + 5.5 cc. of 7 per cent $BaSO_4$; No. 3 = 2 cc. serum + 11 cc. of 7 per cent $BaSO_4$

TABLE 23

The absorption of thermolabil anticomplementary properties from human serum by barium sulphate

AMOUNT OF SERUM (1:10)	UNTREATED SERUM		NO. 1*		NO. 2		NO. 3	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.								
0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.2	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.3	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.4	M.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.5	M.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.6	M.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.7	S.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.8	S.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.9	M.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
1.0	M.H.	C.H.	V.M.H.	C.H.	C.H.	C.H.	C.H.	C.H.
2.0	V.M.H.	C.H.	V.M.H.	C.H.	C.H.	C.H.	C.H.	C.H.
3.0	M.H.	C.H.	M.H.	C.H.	C.H.	C.H.	C.H.	C.H.

* No. 1 = 2 cc. serum + 1.1 cc. of 7 per cent $BaCO_4$; no. 2 = 2 cc. serum + 5.5 cc. of 7 per cent $BaSO_4$; no. 3 = 2 cc. serum + 11 cc. of 7 per cent $BaSO_4$.

In a second set of experiments sera were diluted 1:10 with saline and titrated for antilysins before and after heating; 2 cc. of each serum was then diluted 1:10 with increasing strengths of barium sulphate as 7, 10 and 20 per cent suspensions in saline; the mixtures were incubated for an hour followed by removal of the barium and antilytic tests with the supernatant, diluted serum before and after heating.

The results of experiments conducted in this manner are shown

TABLE 24

The absorption of antilysins from human sera by barium sulphate

AMOUNT OF SERUM (1:10)	UNTREATED SERUM		NO. 1*		NO. 2		NO. 3	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.								
0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.2	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.3	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.4	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.5	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.6	V.M.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.7	S.H.	V.M.H.	V.M.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.8	N.H.	M.H.	M.H.	V.M.H.	V.M.H.	C.H.	C.H.	C.H.
0.9	N.H.	S.H.	S.H.	M.H.	S.H.	V.M.H.	V.M.H.	V.M.H.
1.0	N.H.	S.N.	S.H.	V.M.H.	S.H.	V.M.H.	V.M.H.	V.M.H.
2.0	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.
3.0	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.

* No. 1 = 2 cc. serum + 18 cc. of 7 per cent $BaSO_4$; no. 2 = 2 cc. serum + 18 cc. of 10 per cent $BaSO_4$; no. 3 = 2 cc. serum + 18 cc. of 20 per cent $BaSO_4$.

in tables 24 and 25 and they indicate that barium may remove small amounts of both thermolabil and thermostabil antilysins.

2. The influence of absorptions with kaolin, charcoal and other substances. In these experiments sera were diluted 1:10 and titrated for antilytic activity before and after heating; portions of 20 cc. were then treated with 0.5 gram of the following: Kaolin, silicon, charcoal (wood) and bone ash, previously sterilized; mixtures were made in mortars and after incubation the diluted sera were recovered by centrifugation and filtration through paper and antilytic tests were conducted with each before and after heating.

The results of an experiment of this kind are shown in table 26; kaolin and bone ash usually removed slight amounts of both thermolabil and thermostabil antilysins but not to the same extent as barium sulphate.

In additional experiments anticomplementary sera were divided into five portions of 2 cc. each and kaolin added to the first four in increasing amounts as 0.1, 0.2, 0.3, and 0.4 gram; after thorough mixing, these and the fifth portion (control) were heated at 56°C. for thirty minutes; the kaolin was removed, each serum

TABLE 25

The absorption of antilysins from human luetic sera by barium sulphate

AMOUNT OF SERUM (1:10)	UNTREATED SERUM		No. 1*		No. 2		No. 3	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.								
0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.2	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.4	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.6	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.8	V.M.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
1.0	M.H.	V.M.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
2.0	S.H.	V.M.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
3.0	N.H.	S.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
Control	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

* No. 1 = 2 cc. serum + 18 cc. of 7 per cent $BaSO_4$; no. 2 = 2 cc. serum + 18 cc. of 10 per cent $BaSO_4$; no. 3 = 2 cc serum + 18 cc. of 20 per cent $BaSO_4$.

was diluted 1:10 and this was followed by titrations for thermostabil antilysins. The results of an experiment shown in table 27 indicate that kaolin is capable of removing small amounts of antilysin; with contaminated sera it is highly probable that a part of this result is due to the removal of bacteria by the processes of absorption, centrifugation and filtration.

3. The influence of absorption with washed red blood corpuscles. Since Noguchi has found that red blood corpuscles may absorb thermostabil antilysins from dog, ox and sheep sera and thereby acquire an increased resistance to serum hemolysis, similar experiments have been conducted with anticomplementary human sera.

TABLE 26

The removal of anti-complementary substances from human serum by various substances

AMOUNT OF SERUM (1:10)	UNTREATED SERUM		TREATED SERUM WITH KAOLIN		TREATED SERUM WITH SILICON		TREATED SERUM WITH CHARCOAL		TREATED SERUM WITH BONEASH	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.										
0.1	V.M.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.5	V.M.H.	V.M.H.	C.H.	C.H.	V.M.H.	V.M.H.	V.M.H.	C.H.	C.H.	C.H.
1.0	M.H.	M.H.	V.M.H.	V.M.H.	M.H.	M.H.	M.H.	V.M.H.	M.H.	C.H.
1.5	M.H.	M.H.	M.H.	M.H.	S.H.	M.H.	S.H.	M.H.	S.H.	M.H.
2.0	S.H.	S.H.	S.H.	M.H.	S.H.	S.H.	S.H.	M.H.	S.H.	S.H.
3.0	N.H.	S.H.	S.H.	M.H.	S.H.	S.H.	S.H.	S.H.	N.H.	S.H.
Control	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

Each serum was divided into portions of 0.5 cc. and diluted with 4.5 cc. of a 2.5 per cent suspension of washed sheep cells (1:10) after heating for thirty minutes at 56°C., 60°C., 70°C. and unheated. These mixtures were incubated at 37°C. for three hours and centrifuged; the supernatant fluids (sera diluted 1:10) were titrated for antilysins as was, likewise, a portion of the serum diluted 1:10 and treated as above but without corpuscles, as a control.

The results of the antilytic tests showed that the red blood corpuscles had not removed the antilysins; occasionally with contaminated sera the thermostabil antilysins were removed to a

TABLE 27

The removal of anticomplementary substances from human sera with kaolin

AMOUNT OF SERUM (1:10)	NUMBER OF SERUM				
	No. 1	No. 2	No. 3	No. 4	Control
cc.					
0.1	C.H.	C.H.	C.H.	C.H.	C.H.
0.5	C.H.	C.H.	C.H.	C.H.	C.H.
1.0	C.H.	V.M.H.	C.H.	C.H.	V.M.H.
1.5	S.H.	M.H.	V.M.H.	C.H.	S.H.
2.0	S.H.	S.H.	S.H.	S.H.	N.H.
3.0	N.H.	N.H.	N.H.	N.H.	N.H.

slight extent but no more than could be accounted for by the removal of a portion of the bacteria during the process of centrifuging.

The corpuscles recovered from the sera were re-suspended in steril saline solution and their resistance to serum hemolysin was determined with decreasing amounts of complement. Equal suspensions of untreated cells were titrated at the same time as controls. The results of these titrations showed no differences in the resistance of the corpuscles.

According to these results red blood corpuscles do not absorb the thermolabil or thermostabil antilysins of human sera nor acquire increased resistance to serum hemolysis by contact with them.

4. The influence of neutralization. As previously stated, steril and contaminated sera containing thermolabil antilysin alone or in conjunction with thermostabil antilysins, gradually became more alkaline in reaction to a point where further changes apparently cease. In a series of experiments large volumes of anticomplementary sera were titrated for the degree of alkalinity and hydrochloric acid added to the neutral point. Titrations of such sera heated and unheated before and after neutralization, showed no differences in antilytic titers.

In additional experiments anticomplementary sera were treated with varying amounts of a 4 per cent solution of boric acid in physiological saline solution, but without influencing the antilytic titers of the sera either before or after heating.

5. The influence of filtration. As shown by Muir and Browning (9), filtration of active serum through porcelain removes hemolytic complement; we have been able to corroborate this observation; we have found that active guinea-pig serum passed through a small chemically clean and sterile Kitasato filter is rendered inactive by removal of all traces of hemolytic complement.

Experiments conducted with human sera containing thermolabil and thermostabil antilysins have shown that filtration of a 1:10 dilution of serum through these small earthen filters effectually removes all of the antilysins

In conducting these experiments human sera were diluted 1:10 with physiological saline solution and a portion titrated for antilysins before and after heating; 10 cc. of the remaining portions were passed through the filters by suction and the tests were repeated before and after heating.

The results of one experiment with four sera are shown in tables 28 and 29; in table 28 are shown the results with the sera before filtration and in table 29 the results after filtration.

As shown in these tables filtration through chemically clean and steril Kitasato filters removes thermostabil and thermolabil antilysins from sera diluted 1:10; with filters used more than twice removal is incomplete as likewise with undiluted sera,

owing to the small size of the filters and progressive deterioration following their frequent use.

With sera containing bacteria and thermostabil antilysins filtration of diluted sera through paper and prolonged centrifuging also tends to remove small amounts of antilysin, probably

TABLE 28

The anticomplementary substances in human serum before filtration

AMOUNT OF SERUM (1 : 10)	No. I		No. II		No. III		No. IV	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.								
0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.5	V.M.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
1.0	S.H.	V.M.H.	M.H.	C.H.	C.H.	C.H.	C.H.	C.H.
1.5	S.H.	V.M.H.	M.H.	C.H.	C.H.	C.H.	C.H.	C.H.
2.0	N.H.	V.M.H.	S.H.	V.M.H.	V.M.H.	C.H.	M.H.	C.H.
3.0	N.H.	M.H.	N.H.	M.H.	M.H.	C.H.	C.H.	C.H.
Control	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

TABLE 29

The removal of anticomplementary substances by filtration through Kitasato filters

AMOUNT OF SERUM (1 : 10)	No. I		No. II		No. III		No. IV	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.								
0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.5	C.H.	C.H.	C H.	C.H.	C.H.	C.H.	C.H.	C.H.
1.0	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
1.5	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
2.0	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
3.0	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
Control	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

by the removal of a portion of the bacteria, as shown in table 30; filtration through the Kitasato filters, however, effectually removes all antilysin under the conditions mentioned above.

Of further interest in this connection are the results of additional experiments with emulsions of *Staphylococcus aureas*, which

have shown that filtration removes in large part or entirely the antilytic substances (table 31); however, alcoholic extracts of tissues reinforced with cholesterin used as antigens in the Wassermann test, diluted with saline solution and filtered show no reduction in the antilytic titers (table 32) although the antigenic properties are entirely removed (table 33).

TABLE 30

The removal of antilysins by filtration through Kitasato filters, centrifuging and paper filtration

AMOUNT OF SERUM (1:10)	UNFILTERED SERUM		PORCELAIN FILTERED SERUM		CENTRIFUGALIZED SERUM		PAPER FILTERED SERUM	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
cc.								
0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.5	V.M.H.	C.H.	C.H.	C.H.	V.M.H.	C.H.	C.H.	C.H.
1.0	S.H.	S.H.	C.H.	C.H.	S.H.	C.H.	S.H.	C.H.
1.5	N.H.	S.H.	C.H.	C.H.	S.H.	C.H.	V.S.H.	C.H.
2.0	N.H.	V.S.H.	C.H.	C.H.	N.H.	M.H.	M.H.	M.H.
3.0	N.H.	V.S.H.	C.H.	C.H.	N.H.	M.H.	N.H.	S.H.
Control	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

TABLE 31

The influence of filtration upon the antilytic activity of a broth culture of staphylococcus aureus

BROTH	AMOUNT OF BROTH (UNDILUTED)								
	0.1	0.2	0.4	0.6	0.8	1.0	2.0	3.0	Control
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	
Unfiltered broth.....	V.M.H.	M.H.	M.H.	M.H.	M.H.	M.H.	S.H.	S.H.	C.H.
Filtered broth.....	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	V.M.H.	V.M.H.	C.H.

Filtration of syphilitic sera effects the complete removal of thermolabil and thermostabil antilysins with practically no influence upon the syphilitic antibody concerned in the Wassermann test, if account is taken of the effect upon the test following the removal of the antilysins (tables 34 and 35).

TABLE 32

The influence of filtration on the antilysin in an alcoholic extract of beef heart reenforced with cholesterin

AMOUNT OF ANTIGEN (1:5)	UNFILTERED		FILTERED	
	Unheated	Heated	Unheated	Heated
cc.				
0.1	C.H.	C.H.	C.H.	C.H.
0.2	C.H.	C.H.	C.H.	C.H.
0.4	C.H.	C.H.	C.H.	C.H.
0.6	C.H.	C.H.	C.H.	C.H.
0.8	C.H.	C.H.	C.H.	C.H.
1.0	C.H.	C.H.	C.H.	C.H.
2.0	V.M.H.	V.M.H.	C.H.	V.M.H.
3.0	N.H.	N.H.	V.M.H.	V.M.H.
Control	C.H.	C.H.	C.H.	C.H.

TABLE 33

The influence of filtration upon the antilytic sensitiveness of an alcoholic extract of beef heart reenforced with cholesterin

ANTIGEN (C. B. H.) 1:10	UNFILTERED		FILTERED	
	Unheated	Heated	Unheated	Heated
cc.				
0.05	+2	+2	−	−
0.1	+3	+4	−	−
0.15	+4	+4	−	−
0.2	+4	+4	−	−
0.25	+4	+4	−	−
0.3	+4	+4	−	−
Serum control	−	−	−	−
Hemolytic control	−	−	−	−

TABLE 34

The influence of filtration on the anticomplementary substances in human serum

AMOUNT OF SERUM (1:10)	UNFILTERED		FILTERED	
	Unheated	Heated	Unheated	Heated
cc.				
0.1	C.H.	C.H.	C.H.	C.H.
0.5	C.H.	C.H.	C.H.	C.H.
1.0	V.M.H.	V.M.H.	C.H.	C.H.
2.0	M.H.	M.H.	C.H.	C.H.
3.0	N.H.	N.H.	C.H.	C.H.
Control	C.H.	C.H.	C.H.	C.H.

Additional experiments with dog sera have shown that filtration removes the substance that is responsible for the non-specific complement fixation reactions described by Kolmer and his associates (10). Heating dog serum at 56°C. for thirty minutes tends to increase its antilytic titer and the power of fixing complement in the presence of lipoidal and bacterial antigens; as

TABLE 35

The influence of filtration upon the Wassermann antibody in human serum

AMOUNT OF SERUM (1:10)	UNFILTERED		FILTERED	
	Unheated	Heated	Unheated	Heated
cc.				
0.1	+1	–	–	–
0.2	+3	+2	–	–
0.4	+4	+4	+1	–
0.6	+4	+4	+1	+1
0.8	+4	+4	+4	+2
1.0	+4	+4	+4	+2
2.0	+4	+4	+4	+3
Serum control, 2.0	+3	+3	–	–
Antigen control	–	–	–	–
Hemolytic control	–	–	–	–

TABLE 36

The influence of filtration on non-specific complement fixation by heated dog serum

AMOUNT OF DOG SERUM (1:10)	TYPHOID ANTIGEN (1:10)	COMPLEMENT (1:20)	ANTISHEEP HEMOLYSIN UNIT	SHEEPS CORPUSCLES (2.5 PER CENT.)	RESULT	
					Unfiltered	Filtered
cc.	*cc.*	*cc.*		*cc.*		
0.1	0.1	1	2	1	C.H.	C.H.
0.2	0.1	1	2	1	C.H.	C.H.
0.4	0.1	1	2	1	C.H.	C.H.
0.6	0.1	1	2	1	C.H.	C.H.
0.8	0.1	1	2	1	V.M.H.	C.H.
1.0	0.1	1	2	1	M.H.	C.H.
2.0	0.1	1	2	1	M.H.	C.H.
S.C.*						
2.0	0	1	2	1	C.H.	C.H.
A.C.	0.1	1	2	1	C.H.	C.H.
H.C.	0	1	2	1	C.H.	C.H.

* S.C. = serum control; A.C. = antigen control; H.C. = hemolytic control.

shown in table 36, filtration removes the substance from dog sera that is capable of yielding non-specific complement fixation reactions with bacterial antigens.

SUMMARY AND CONCLUSIONS

1. Anticomplementary substances (antilysins) in human sera may be divided into two kinds: (a) those removed by heating at 56°C. (thermolabil antilysin) and (b) those not removed by heating (thermostabil antilysin).

2. Human sera do not develop antilysins as a result of heating, as may occur with rabbit, dog and mule sera.

3. Steril sera develop thermolabil but not thermostabil antilysins. Steril sera kept at 37°C. may develop these thermolabil antilysins in from three to seven days; at room and lower temperatures (0 to 2°C.), longer periods are required.

4. Fresh sera rapidly dried in filter paper do not develop anticomplementary properties.

5. Human sera containing various bacteria and particularly staphylococci rapidly develop thermolabil and thermostabil antilysins.

6. Staphylococci alone rather than their products elaborated during cultivation in broth and serum, produce the antilytic effects of contaminated serum.

7. Large amounts of hemoglobin in salt solution and serum, steril and contaminated, exert anticomplementary activities before and after heating. Traces of hemoglobin are usually free of these antilytic effects.

8. Steril and contaminated sera containing antilysins gradually become alkaline in reaction; neutralization of this alkali with hydrochloric acid does not remove the antilytic activities of the sera.

9. Definite changes in the hydrogen ion concentration of steril and contaminated human serum before and after the development of antilysins, could not be determined with colorimetric methods.

10. Steril human sera containing thermolabil antilysin showed a slight increase of total protein and especially of the globulin

fraction, as determined by the refractometric method of Robertson; contaminated sera containing thermolabil and thermostabil antilysins showed a marked increase of total protein and especially of the albumin fraction, probably due in part to the presence of bacteria. Anticomplementary sera passed through a Kitasato filter which removes the antilysins, showed a reduction in total protein and especially of the globulin fraction. Heating anticomplementary sera at 56°C. for thirty minutes, which reduced the content of antilysins, had no appreciable influence upon the protein constituents.

11. The removal of ether soluble lipoids from anticomplementary human sera did not remove the thermolabil and thermostabil antilysins.

12. The antilysins of human sera are closely allied with the protein constituents and especially the globulin fraction.

13. Absorption of human sera with barium sulphate tends to remove a portion of thermolabil and thermostabil antilysins; kaolin, bone ash and charcoal also remove antilysins, but to a lesser degree.

14. Absorption of human sera with washed erythrocytes does not remove thermolabil or thermostabil antilysins as may occur with the thermostabil antilysins of dog serum; erythrocytes exposed to the antilysins of human serum do not acquire an increased resistance to serum hemolysis.

15. Filtration of diluted human sera through new, chemically clean and steril Katasato filters removes all thermolabil and thermostabil antilysin; likewise the filtration of diluted heated dog serum removes the substances responsible for non-specific complement fixation reactions. Filtration of the sera of luetic persons has practically no influence upon the antibody concerned in the Wassermann reaction.

16. Filtration of freshly prepared bacterial antigens removes the antilysins; filtrations of antigens for the Wassermann test as alcoholic extracts of beef heart reinforced with cholesterin diluted 1:20 with saline solution, does not remove the antilysin but completely removes the antigenic substance.

I beg to express my appreciation of the kindness of Professor Kolmer for outlining the experiments and technic of this investigation and for his supervision of the work; also to Dr. Charles Weiss, for aid in conducting the hydrogen ion determinations, and to Dr. Hatai and Dr. Toyama for aid in the refractometric work.

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EXPERIMENTS ON THE REMOVAL OF HEMAGGLUTININ FROM RABBIT ANTIHUMAN SERUM

JOSEPH E. SANDS AND LYLE B. WEST

From the laboratory of experimental pathology of the University of Pennsylvania

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In conducting the complement fixation test for syphilis with an antihuman hemolytic system, agglutination of the erythrocytes not infrequently interferes with hemolysis and the frequency and intensity of agglutination constitutes a drawback in the usefulness of the antihuman system.

One of the sources of this agglutinin is the immune rabbit hemolytic serum and at the suggestion of Professor Kolmer, we have conducted a series of experiments with rabbit immune sera with the object of determining whether or not agglutinin for human cells may be removed without disturbing the hemolysin content. Professor Kolmer has been of the opinion that drying antihuman rabbit serum on paper after the method of Noguchi, resulted not only in facilitating the preservation and manipulation of the hemolysin but also in the deterioration of agglutinin without commensurate destruction of hemolysin and that this constituted an important reason for the use of paper amboceptor in complement fixation tests when an antihuman hemolytic system is employed. Our experiments have supported this view and in addition they have shown that filtration also tends to remove the agglutinin from a hemolytic serum constituting an observation of considerable theoretical and possibly of some practical value.

TECHNIC

In conducting hemolysin titrations one cubic centimeter of dilutions of heated rabbit hemolytic serum varying from 1:10 to 1:120 were placed in a series of test tubes; 0.1 cc. of a 1:10

dilution of guinea-pig complement, 0.5 cc. of a 1 per cent suspension of washed human cells and 0.4 cc. of salt solution were added and incubation was conducted in a water bath at 38°C. for one hour, the readings being made after the mixtures had stood over night in a refrigerator.

The agglutination tests were conducted by placing in a series of test tubes 1 cc. of dilutions of heated rabbit hemolytic serum varying from 1: 10 to 1: 960 together with one cubic centimeter of a one per cent suspension of washed human cells; incubation took place in a water bath at 38°C. for one hour and the readings were made the following morning after the tubes had stood in a refrigerator.

In presenting the results the titer of each serum is given as the actual amount of serum as such or as calculated with a solution of dried serum, producing agglutination or hemolysis in a constant volume of 2 cc. and in the time specified above.

A number of different rabbit hemolytic sera were prepared by immunizing with washed human cells after various methods (1) and employed in conducting these experiments.

THE INFLUENCE OF DRYING UPON HEMAGGLUTININ AND HEMOLYSIN

These experiments were carried out with two methods:

(*a*) Measured amounts of each immune serum were rapidly dried by fanning with cold air in a measured amount of Schleicher and Schull's paper no. 597 and the amount of serum per square millimeter of paper was estimated; hemolysin and agglutinin titrations were then conducted with increasing amounts of paper and the results were compared with tests conducted with varying amounts of fluid serum.

(*b*) In the second method, measured amounts of serum were rapidly dried by fanning with cold air in weighed dishes and the amount of dried product was calculated per cubic centimeter of serum. This amount was then weighed and dissolved in 10 cc. of physiological salt solution yielding opalescent solutions of dried serum equal, or very nearly so, to a 1: 10 dilution of

fluid serum secured by diluting 1 cc. of serum with 9 cc. of physiological salt solution. Comparative hemolysin and agglutination tests were then conducted at the same time and with the same corpuscles and complement.

The results of a series of these experiments are summarized in tables 1 and 2.

TABLE 1

The influence of drying rabbit antihuman serum in filter paper upon agglutinin and hemolysin

SERA	INFLUENCE ON AGGLUTININ		INFLUENCE ON HEMOLYSIN	
	Titer of serum	Titer of serum after drying	Titer of serum	Titer of serum after drying
1	0.0013	0.0015	0.016	0.015
2	0.0033	0.0144	0.04	0.01
3	0.0041	0.006	0.06	0.015
4	0.00125	0.003	0.0166	0.013
5	0.00083	0.001	0.01	0.008
6	0.008	0.036	0.016	0.009
7	0.002	0.007	0.01	0.01
8	0.0125	0.02	0.01	0.009

TABLE 2

The influence of drying rabbit antihuman serum in dishes upon agglutinin and hemolysin

SERA	INFLUENCE ON AGGLUTININ		INFLUENCE ON HEMOLYSIN	
	Titer of serum	Titer after drying	Titer of sernm	Titer after drying
1	0.0013	0.02	0.016	0.016
2	0.0033	0.015	0.04	0.04
3	0.0041	0.035	0.06	0
4	0.00125	0.006	0.0166	0.016
5	0.00083	0.003	0.01	0.01
6	0.008	0.05	0.016	0.01
7	0.002	0.003	0.01	0.009
8	0.0125	0.01	0.01	0.0085

As shown in tables 1 and 2, drying sera in filter paper and in dishes usually resulted in a well defined reduction of the agglutinins; the hemolysins showed no appreciable deterioration but rather an enhanced hemolytic activity, which we ascribed to the removal of the inhibiting influence of the agglutinins.

THE INFLUENCE OF FILTRATION UPON HEMAGGLUTININ AND HEMOLYSIN

These experiments were conducted by filtering 5 to 10 cc. of each immune serum undiluted and diluted 1: 10 with physiological salt solution through Kitasato and Chamberland candle filters and comparing the agglutinin and hemolysin content with unfiltered serum tested at the same time with the same indicator antigen and complements.

The results of a number of experiments are given in table 3.

As shown in table 3, filtration through Kitasato and Chamberland candles usually removed a large amount of hemagglutinin

TABLE 3

The influence of filtration of rabbit antihuman sera upon agglutinin and hemolysin

SERA	FILTER	INFLUENCE ON AGGLUTININ		INFLUENCE ON HEMOLYSIN	
		Before filtration	After filtration	Before filtration	After filtration
1 (1: 10)	Kitasato	0.0013	0.006	0.016	0.016
2 (1: 10)	Kitasato	0.0033	0.01	0.04	0.03
3 (1: 10)	Kitasato	0.0041	0.0125	0.06	0.025
4 (1: 10)	Kitasato	0.00125	0.033	0.0166	0.008
5 (1: 10)	Kitasato	0.00083	0.0125	0.01	0.03
6 (1: 10)	Kitasato	0.002	0.006	0.01	0.008
7 (undiluted)	Kitasato	0.002	0.003	0.01	0.01
8 (1: 10)	Chamberland	0.0125	0.005	0.01	0.008

without appreciable influence upon the hemolysins. Best results were observed with sera diluted 1: 10 with physiological salt solution and with new filters cleansed with sterile distilled water and sterilized. Filters used more than three times with proper cleansing and burning in a blast lamp between filtrations became so porous that slight or no influence was exerted upon the filtered sera.

Not infrequently the hemolytic titer of a serum was increased as a result of filtration and we have ascribed this to the removal of the inhibiting influence of agglutinin.

The first portion of the filtrate always showed a greater reduction of agglutinin than following portions; serum 5 for example,

showed an agglutinin titer of 1:1200 before filtration, 1:160 with the first 5 cc. of filtered serum and 1:400 with the second 5 cc. When 10 cc. of salt solution were then drawn through the empty filter some of the agglutinin was withdrawn, inasmuch as this salt solution showed an agglutinin titer of 1:200 and a trace of hemolysin.

THE INFLUENCE OF BARIUM SULPHATE ABSORPTION UPON HEMAGGLUTININ AND HEMOLYSIN

Wechselmann and Lange having shown that absorption of syphilitic sera with barium sulphate tends to remove certain constituents and increasing the degree of complement fixation in the Wassermann reaction, we have conducted additional experiments upon the influence of barium sulphate upon rabbit antihuman sera; Noguchi and Bronfenbrenner (2) have shown that barium sulphate tends to remove natural antisheep hemolysin from human sera and may in this manner increase the delicacy of the Wassermann reaction as shown by Wechselmann and Lange.

Our experiments were conducted by adding to 1 cc. of immune serum increasing amounts of a seven per cent suspension of barium sulphate and incubating for one hour with frequent shakings. Physiological salt solution was then added to make the final dilution of serum 1:20 and each mixture centrifuged and filtered through paper to remove the barium. The exact proportions were as follows:

1 cc. serum plus 1.1 cc. of 7 per cent barium plus 17.9 cc. salt solution
1 cc. serum plus 5.5 cc. of 7 per cent barium plus 13.5 cc. salt solution
1 cc. serum plus 11.0 cc. of 7 per cent barium plus 8.0 cc. salt solution
1 cc. serum plus 19.0 cc. of 7 per cent barium

Agglutinin and hemolysin titrations were then conducted with several immune sera before and after absorption with barium sulphate; the results are given in table 4.

As shown in table 4, barium sulphate removes from rabbit antihuman serum varying amounts of hemagglutinin; 1.1 cc. of

a 7 per cent suspension of this substance removes from 1 cc. immune serum almost as much agglutinin as the larger amounts.

With two sera the hemolytic titers were appreciably increased as the result of absorption with barium; with a third serum (no. 6) a decrease in hemolytic activity was observed due presumably to removal of hemolysin. We are unable to explain the increase of hemolytic activity of sera 2 and 3; the suspension of barium itself was not hemolytic as tested by centrifuging and filtering a portion of the 7 per cent suspension used and testing the filtrate with the same suspensions of washed human cells employed in these tests.

TABLE 4

The influence of barium sulphate absorption of antihuman rabbit serum upon hemagglutinin and hemolysin

SERA	UNTREATED		1.1 CC. OF BARIUM		5.5 CC. OF BARIUM		11 CC. OF BARIUM		19 CC. OF BARIUM	
	Agglutinin	Hemolysin	Agglutinin	Hemolysin	Agglutinin	Hemolysin	Agglutinin	Hemolysin	Agglutinin	Hemolysin
2	1:300	1:25	1:80	1:20	1:80	1:40	1:80	1:30	1:80	1:30
3	1:240	1:16	1:80	1:50	1:80	1:50	1:60	1:50	1:60	1:50
6	1:120	1:60	1:60	1:25	1:60	1:25	1:50	1:20	1:50	1:20

THE INFLUENCE OF ABSORPTION BY HUMAN CELLS UPON HEMOLYSIN AND AGGLUTININ

It is well known that human cells added to antihuman immune serum will absorb the specific hemolysin and agglutinin, but inasmuch as agglutination usually appears earlier than hemolysis in complement fixation tests conducted with an antihuman hemolytic system, we have conducted several experiments by adding to 1 cc. of heated immune serum 1 cc. of washed packed human cells and centrifuging after incubation in a water bath at 55°C. for fifteen minutes to determine what proportion of hemagglutinin and hemolysin were removed from the serum under these conditions.

The results of experiments with three sera are shown in table 5.

As shown in table 5 absorption with human cells removed agglutinin and hemolysin but by adding a large volume of cells and removing them after a short exposure in the serum as conducted in our experiments, a proportionately larger amount of agglutinin was apparently removed.

TABLE 5

The influence of absorption with human erythrocytes of rabbit antihuman sera upon hemagglutinin and hemolysin

SERUM	BEFORE ABSORPTION		AFTER ABSORPTION	
	Agglutination	Hemolysis	Agglutination	Hemolysis
1	1:750	1:60	1:50	1:10
2	1:300	1:25	1:25	1:15
3	1:240	1:20	1:25	1:20

THE INFLUENCE OF HYPERTONIC SOLUTION OF SODIUM CHLORID UPON THE HEMAGGLUTININS AND HEMOLYSINS IN RABBIT ANTIHUMAN SERUM

Owing to the well known influence exerted by varying concentrations of sodium chlorid upon the agglutination of bacteria and also upon opsonins and the phenomenon of phagocytosis, we have also conducted a series of experiments with three immune sera for the purpose of determining the influence of hypertonic solutions of sodium chlorid upon the hemagglutinins and hemolysins.

Solutions of chemically pure solium chlorid in distilled water were prepared in concentrations varying from 0.8 to 5 per cent and 10 cc. of each placed in a series of test tubes; to each tube was added 0.1 cc. of washed packed human cells giving approximately a one per cent suspension in each.

Agglutination tests were conducted by placing 1 cc. of each suspension in corresponding test tubes and adding 1 cc. of a dilution of immune serum in distilled water known to contain two units of agglutinin, which doubled each dilution of sodium chlorid. After mixing and incubating for one hour in a water

bath at 38°C. the results were read after standing in a refrigerator over night.

Hemolysin tests were conducted by placing 1 cc. of each corpuscle suspension in corresponding tubes and adding 0.5 cc. of a dilution of immune serum in distilled water known to contain two units of hemolysin, 0.1 cc. of a 1:10 dilution of guinea-pig serum complement in water and 0.4 cc. of distilled water, which resulted in doubling each solution of sodium chlorid. The results were read after incubation in a water bath for one hour followed by refrigeration over night.

The results of several experiments are shown in table 6.

TABLE 6

The influence of hypertonic solutions of sodium chlorid upon hemagglutinins

SERUM	UNIT OF SERUM*	AMOUNT OF SERUM USED	RESULTS OF AGGLUTINATION TESTS IN THE PRESENCE OF INCREASING PERCENTAGES OF SODIUM CHLORID															
			1%	1.1%	1.2%	1.3%	1.4%	1.5%	1.6%	1.7%	1.8%	1.9%	2%	2.1%	2.2%	2.3%	2.4%	2.5%
1	1 cc. of 1:750	1 cc. of 1:350	+†	+	+	+	+	+	±	±	±	±	±	±	±	±	±	±
2	1 cc. of 1:300	1 cc. of 1:150	+	+	+	±	±	±	±	±	±	±	±	±	±	±	±	±
3	1 cc. of 1:240	1 cc. of 1:120	+	+	±	±	±	±	±	±	±	±	±	±	±	±	±	−

* Titrated in the presence of 0.85 per cent sodium chlorid.

† + = agglutination, ± partial agglutination.

As shown in table 6, concentrations of sodium chlorid in a final dilution of 1.6 per cent and higher tend to protect human erythrocytes against hemagglutinin, but do not entirely prevent agglutination in solutions of as high as 2.5 per cent unless the amount of agglutinin in the serum is relatively small, as in serum 3.

As shown in table 7 hemolysis is interfered with by final dilutions of sodium chlorid ranging from 1.7 or 2 per cent and higher; for this reason hypertonic solutions of sodium chlorid are of slight practical value for reducing the influence of hemagglutinins in complement fixation tests in which an antihuman hemolytic system is employed.

TABLE 7

The influence of hypertonic solutions of sodium chlorid upon hemolysis

SERUM	UNIT OF SERUM	DOSE OF SERUM USED	RESULTS OF HEMOLYSIN TESTS IN THE PRESENCE OF INCREASING PERCENTAGES OF SODIUM CHLORID							
			1%	1.1%	1.2%	1.3%	1.4%	1.5%	1.6%	1.7%
	cc.	*cc.*								
1	0.016	0.03	C.H†	C.H	C.H	C.H	C.H	C.H	C.H	M.H
2	0.04	0.08	C.H	C.H	C.H	C.H	C.H	C.H	C.H	C.H
3	0.04	0.08	C.H	C.H	C.H	C.H	C.H	C.H	C.H	M.H

SERUM	UNIT OF SERUM*	DOSE OF SERUM USED	RESULTS OF HEMOLYSIN TESTS IN THE PRESENCE OF INCREASING PERCENTAGES OF SODIUM CHLORID						
			1.8%	1.9%	2%	2.1%	2.2%	2.3%	2.5%
	cc.	*cc.*							
1	0.016	0.03	S.H	N.H	N.H	N.H	N.H	N.H	N.H
2	0.04	0.08	M.H	S.H	N.H	N.H	N.H	N.H	N.H
3	0.04	0.08	S.H	N.H	N.H	N.H	N.H	N.H	N.H

* Titrated in the presence of 0.85 per cent sodium chlorid.

† C.H = complete hemolysis; M.H = marked hemolysis; S.H = slight hemolysis; N.H = no hemolysis.

CONCLUSIONS

1. Owing to the tendency for retardation or prevention of hemolysis of human erythrocytes by rabbit antihuman serum, due to the presence of hemagglutinin for these cells, experiments were conducted for the purpose of determining whether practical methods for the removal of the hemagglutinin could be devised.

2. Drying serum upon filter paper by the method of Noguchi, or in evaporating dishes was found to result in distinct destruction of hemagglutinin with slight or no destruction of specific hemolysin. The use of paper amboceptor therefore in complement fixation tests employing the antihuman hemolytic system is advisable for this reason in addition to being a satisfactory method for the preservation and manipulation of this constituent.

3. Filtration of immune sera and especially 1:10 dilutions through satisfactory Kitasato and Chamberland filters removes a large amount of hemagglutinin with slight or no depreciation in hemolytic activity; hemolytic activity may be increased, due presumably, to the removal of the hemagglutinins.

4. Absorption of rabbit antihuman serum with barium sulphate removed large amounts of hemagglutinin; the removal of specific hemolysin was irregular and occasionally the hemolytic activity of the treated serum was increased.

5. Absorption of rabbit antihuman serum with a large volume of washed human erythrocytes for a short period tends to remove a proportionately larger amount of hemagglutinin than hemolysin.

6. Solutions of sodium chlorid in final dilution of 1.5 per cent and higher tend to prevent hemagglutination; dilutions of 1.7 or 2 per cent and higher tend to interfere with hemolysis so that the use of hypertonic solutions of sodium chlorid are of no practical value in preventing hemagglutination in complement fixation tests in which an antihuman hemolytic system is employed.

We wish to express our sincere thanks to Professor Kolmer for his advice and directions and to Miss Anna Rule for aid in conducting some of the experiments.

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A NEW METHOD OF TESTING ANTITYPHOID SERUM

YOSHIMOTO FUKUHARA AND MASAAKI YOSHIOKA

From the Pathological-Bacteriological Institute in Osaka, Japan (Director: Professor A. Sata)

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It is well known that no antibodies have heretofore been discovered in antibacterial (antiinfectious) sera, whose quantitative determination could be used as a measure of therapeutic value. Nevertheless, practical experience has shown that there are a number of antiinfectious sera which, notwithstanding their variable content in one or another of the known antibodies, are practically useful and can be quantitatively titrated with the use of animals. This titration can be carried out by the preliminary protective injection of the serum followed immediately or later by the injection of living bacteria. However, since the time of Pfeiffer it has been the custom to inject the serum in varying quantities combined with ten lethal doses of a living virulent culture of the bacteria. The injections are made into the peritoneal cavity. The quantity of serum which was just able to protect the animal against ten lethal doses of bacteria was designated by Pfeiffer as the titer or immunity unit of the serum.

For this test in typhoid it has been customary to use strains of such virulence that a fifth to a tenth of one *oese* could kill a guinea-pig in twenty-four hours after peritoneal injection. The varying virulence of the cultures offered a source of difficulty in making the test. As to the cause of this variability we shall speak later.

The protocols of an experiment presented in tables 1a, 1b and 1c demonstrate the disadvantages of the usual method of titrating an antibacterial serum.

In carrying out this test we chose the procedure given by Pfeiffer for testing anti-cholera serum. One cubic centimeter of the diluted immune serum of goat A was stirred in a test-tube with ten lethal doses of three different living virulent strains of typhoid organisms (Monoyama 2, Monoyama 3 and Takayama PI) from an eighteen to twenty hour agar culture and this suspension was injected intraperitoneally into a guinea-pig of about 250 grams weight.

TABLE 1A

The testing of goat's serum A with strain Momoyama 2; lethal dose, 0.125 oese

SERUM DILUTION	GUINEA-PIG WEIGHT	RESULTS
	grams	
1:20	230	Lived
	230	Lived
	260	Lived
	245	Died after 15 hours
1:22	230	Died after 15 hours
	230	Lived
1:24	250	Died after 15 hours
	240	Died after 15 hours
1:27	235	Died after 17 hours
	230	Died after 17 hours
1:32	230	Died after 17 hours
	230	Died after 17 hours

It is seen that the titer of the goat's serum is about 1–20 with strain Monoyama 2; 1–25 with the strain Takayama PI and 1–55 with the strain Monoyama 3. In other words, 0.04 cc. of the immune serum protects against ten lethal doses of strain Takayama PI; 0.018 cc. protects against ten lethal doses of strain Monoyama 3 and 0.05 cc. protects against ten doses of Monoyama 2.

The method of testing antityphoid sera that is illustrated in the foregoing table is obviously unreliable, since the result of the determination of protective value depends, here, on the strain of the bacteria employed in the test.

The experiment demonstrates clearly the need of a constant standard of protective value, which could only be established with the use of a constant unit of the infectious material; that is, of the typhoid culture. It occurred to us that the principle

TABLE 1B

The testing of goat's serum A with strain Takayama PI; lethal dose, 0.11 oese

SERUM DILUTION	GUINEA-PIG WEIGHT	RESULTS
	grams	
1:10	230	Lived
	230	Lived
	235	Lived
	235	Lived
1:20	240	Died after 18 hours
	250	Lived
	235	Lived
	280	Lived
1:25	250	Lived
	235	Lived
1:30	245	Died after 18 hours
	260	Lived
	255	Lived
	270	Lived
	260	Died after 18 hours
1:35	240	Lived
	250	Lived
	220	Died after 18 hours
1:36	255	Lived
	230	Died after 17 hours
1:37	230	Lived
	230	Died after 18 hours

employed in the testing of antitoxic sera might be applicable, also, to the testing of antibacterial sera and, as will be presently shown, our anticipations in this regard have been realized.

Our first step in applying this principle was the selection of a standard protective unit, which, as in the case of the standard

units of antitoxin, is necessarily an arbitrary one. The unit was arrived at in the following manner. Varying amounts of antityphoid serum 180 were mixed with ten lethal doses of an agar culture of the typhoid strain Takayama and these mixtures

TABLE 1c

The testing of goat's serum A with strain Momoyama 3; lethal dose, 0.1 oese

SERUM DILUTION	GUINEA PIG WEIGHT	RESULTS
	grams	
1:45	240	Died after 18 hours
	240	Lived
1:50	230	Lived
1:55	230	Lived
	240	Lived
1:58	230	Died after 18 hours
	245	Lived
1:60	225	Died after 18 hours
	225	Died after 18 hours
1:63	240	Lived
	230	Lived
	230	Lived
	245	Lived
1:64	245	Lived
	235	Lived
1:65	230	Died after 18 hours
	235	Died after 18 hours
	230	Lived
1:66	245	Lived
	250	Died after 18 hours

were injected intraperitoneally into guinea-pigs. The amount of serum which just sufficed to protect the animal from death was taken as the unit and it was found, as a result of repeated tests, that 1 gram of the dry serum contained 4410 such protective units. The protocols of these tests are presented in

TABLE 2

Testing of the original standard serum 180 PS

SERUM DILUTION	GUINEA PIG WEIGHT	RESULTS
	grams	
1:500	230	Lived
	255	Lived
1:600	240	Lived
1:1000	230	Lived
1:2000	230	Lived
1:4000	250	Lived
	230	Died after 49 hours
1:4200	240	Lived
	250	Lived
1:4300	250	Lived
1:4400	250	Lived
	250	Lived
1:4410	250	Lived
	230	Lived
1:4415	230	Died after 20 hours
1:4420	230	Died after 21 hours
	230	Died after 2 days
1:4440	230	Lived
	250	Died after 46 hours
1:4460	240	Died after 21 hours
	240	Lived
1:4480	230	Lived
	240	Died after 27 hours
1:4500	240	Died after 20 hours
	235	Died after 20 hours
1:50000	230	Died after 20 hours
	230	Died after 20 hours

table 2. The minimal lethal dose of the strain employed was about $\frac{3}{4}$ *oese*. It was found advisable, in order to exclude individual differences in the experimental animals, to test the critical amounts on several animals. In measuring the amount of the bacteria we have used the bacteriometer of Rosenthal, which we have somewhat modified (bacteriometer of Fukuhara). The number of the bacteria is calculated from the graduations on the capillary tube containing the bacterial sediment.

The standard antityphoid protective unit having been thus established, it was necessary to see whether, in the standardization of the antitoxic sera, a constant measure of protective power could be determined in the various typhoid cultures, with the use of the arbitrary standard protective unit. In investigating this question, we have adopted the conception of L_+ and the L_0 dose. The L_0 dose we have taken to be the largest amount of the living bacteria which when mixed with the protective unit and intraperitoneally injected did not cause the death of a guinea-pig of 250 to 300 grams weight; the L_+ dose has been considered to be the smallest amount of typhoid bacteria which when mixed with the protective unit of antiserum and injected intraperitoneally into a guinea-pig of 250 to 300 grams weight caused the death of the animal in the course of twenty-four hours. The protocols of the determination of these two limits in three different strains of typhoid bacteria are presented in table 3.

It is seen that both of these values differ in the different strains as follows:

STRAIN	L_+	L_0
	graduations	*graduations*
Momoyama 2	7	4.3
Takayama PI	7.2	5.0
Takayama	12.0	9.7

The L_+ and L_0 doses having been determined for the strain of bacteria to be employed, it remained to be seen whether these doses can be used, in turn, to determine the protective

unit of an antityphoid serum. This question was pursued in a manner analogous to the method employed in testing antitoxic sera. The L_+ dose (or the L_0 dose) was mixed with different amounts of the serum to be tested and the mixtures were in-

TABLE 3

Determination of the L_+ *and* L_0 *dose*

AMOUNT OF BACTERIA IN GRADUATIONS	TAKAYAMA	MOMOYAMA 2	TAKAYAMA PI
14.0	T T		
13.0	T T		
12.0	T T		
11.5	O O		
11.0	T O		
10.0	T O		
9.7	O O		
9.4	O O		
9.0	O O		
8.0	O O		T T
7.6	O O	T T T T T T	T T
7.4		T T T O	
7.2		T T	T T
7.0		T T T O	T O
6.8		T O	T O
6.4		T O	T O O O
6.2			T T
6.0		T T T O	O O O T
5.8		T T O O	
5.6		T O T O	T T
5.0		T T	O O O T
4.9			O O
4.5		T O T O	O O
4.4		T O	
4.3		O O T O	
4.2		O O	
4.1		O O	
4.0		O O	O O
3.9		O O	
3.5		T O O O	O O
3.0		T O O O	
2.5		O O	

The minimal lethal dose of the respective strains were: Takayama, 1.2 graduations; Momoyama 2, 1.7 graduations; Takayama PI, 1.1 graduations.

T indicates the death of the animal within twenty-four hours.

O indicates that the animal survived the injection.

jected intraperitoneally into guinea-pigs. The protocols of such a test are presented in tables 4a and 4b.

It is seen that the method possesses considerable reliability. Notwithstanding the wide variation in the L_+ dose of the different strains of bacteria, the determined protective value of the antisera was practically the same whichever strain was used in the test. As a result of numerous experiments we have come to the conclusion that the use of the L_0 dose is not practical. We prefer, therefore, to test the antisera with the L_+ dose.

TABLE 4A

The testing of typhoid antiserum 180B with the use of the L_+ dose

SERUM DILUTION	TAKAYAMA P	MOMOYAMA 2	637
1:300	T T		
1:200	T O		
1:100	T T		
1: 50	T T		
1: 45	T T	T O	
1: 40	T T	T T	T T
1: 36	T T	T T	
1: 31	T T		
1: 30	T O	T O	T O
1: 25	T T	T T	T T
1: 20	T T	T T	T T
1: 19	T T	T T	T T T O
1: 18		T T	T T T O
1: 17	T T	T O	T T
1: 16	O O	T O	T O
1: 15	T O	T O	T O
1: 10	O O	T O	O O

The L_+ dose of the three strains was: Takayama P, 7.3 graduations; Momoyama 2, 7.6 graduations; 637, 55 graduations.

To recapitulate, our method is as follows: with the use of an arbitrarily selected protective unit of an antityphoid serum, which is preserved in the dry state as a standard antiserum, the L_+ dose of a typhoid culture is determined; with this L_+ dose is then determined the protective unit of the serum to be tested; that is, the largest amount of the serum which, when mixed with the L+ dose of the bacteria will not prevent the death of the test animal within 24 hours. If, for instance, this amount should

be found to be 0.001 cc. then, naturally, 1 cc. of the serum contains 1000 protective units.

Although the use of the L_0 dose in testing antityphoid serum was found to be unserviceable, nevertheless, this dose can be used and the same result can be obtained with it as with the L_+ dose. This is shown by an experiment, the results of which are presented in table 5.

TABLE 4B

The testing of typhoid antiserum 80B with the use of the L_+ dose

SERUM DILUTION	MOMOYAMA 2	TAKAYAMA P	637
1:13000	T T		
1:12900	T T		
1:12700	T T	T O	T T
1:12500	T T	T T	T T
1:12300	T T	T T	T T
1:12100	T T	T T	T T
1:12000	T T	T T	T T
1:11980	T T	T O	T O
1:11960	T O	T O	T T
1:11940	T T	T O	T O
1:11920	T O	T O	T T
1:11900	T O		T O
1:11700	T O		O O
1:11500	T T	T O	T T
1:11300	T O		
1:11100	T O	T O	T O
1:11000	T T		
1:10500	O O		
1:10000	T O		
1: 7000	O O	O O	T O

The L_+ dose of the three strains were: Momoyama 2, 3.98 graduations; Takayama P, 4.42 graduations; 637, 54 graduations.

The standard serum was preserved in the dry state in Ehrlich tubes. When a new standard serum is to be tested, a tube of the old standard serum is opened and the contents are diluted with glycerin-water so that 1 cc. of the fluid contains exactly 10 protective units. With this diluted serum the L_+ dose of a typhoid bacterial culture is determined and this dose is, in turn, employed to estimate the protective value of the new standard serum.

In order to aid in the general adoption of a uniform standard protective unit, the advantages of which are the same as those of the antitoxin units, our institute will be glad to supply standard serum on request.

In the L_+ dose of typhoid bacteria we possess a uniform criterion for the determination of the protective value of an antityphoid serum. Naturally, any strain of typhoid bacteria could be used for testing. The virulence control is not necessary in every test. It would be also unnecessary to increase the virulence of the strain by animal passage, if this were possible. Even the strain 637, 4 *oese* of which could not always kill a guinea-

TABLE 5

The testing of antityphoid serum 180B with the L_0 *dose of bacteria*

SERUM DILUTION	TAKAYAMA P	MOMOYAMA 2
1:35	T T	
1:30	T T	
1:25	T O	
1:22	T O	
1:19		T O
1:18	T T	T O
1:17	O O	O O
1:16		O O
1:15	O O	

The L_0 dose of the two strains was: Takayama P, 4.9 graduations; Momoyama 2, 4.3 graduations.

pig, was found to be as available for the testing of antityphoid sera as other virulent strains. This fact in itself speaks for the practical value of our method of testing antityphoid sera.

It would be practically advantageous to use a culture which would maintain its properties for a considerable time. However, the typhoid cultures constantly diminish in virulence. This we have found to be especially true if the cultures are kept in the ice-box without being transplanted. The diminution of the virulence under such conditions is shown in tables 6a and 6b. Even here, however, the toxicity of the culture remains fairly constant for one to two months. In order to prevent considerable variations in the test doses, it seems advisable to transplant

the cultures every three to six weeks and to preserve the cultures at low temperature.

We have found that the falling off of the virulence of typhoid cultures, which occurs even when the cultures are regularly transplanted, cannot be prevented by animal passage. The constancy of the L_+ dose cannot be maintained by this means. This fact is shown in a series of determinations made over a period of a year and a half, the results of which are presented in table 7.

TABLE 6A

Variations in the lethal and L_+ dose of the strain Momoyama 2

AFTER DAYS	LETHAL DOSE	L_+ DOSE
	graduations	*graduations*
21	1.07	1.80
40	1.00	1.80
60	1.00	2.00
100	1.22	2.60
130	1.20	About 3.00
150	1.70	7.60

TABLE 6B

Variations in the lethal and L_+ dose of the strain Takayama P

AFTER DAYS	LETHAL DOSE	L_+ DOSE
	graduations	*graduations*
21	0.53	3.10
40	0.57	3.30
60	0.58	3.30
100	0.68	4.20
130	0.70	4.40

Pfeiffer and his pupils and also Strong came to the conclusion, as a result of their studies, that the binding power, virulence and immunizing power of cholera cultures are parallel properties. Wassermann, Hetsch and Kutscher, Petterson, Meinicke, Jaffe and Flemming, on the other hand, were unable to find such a parallelism in cultures of typhoid and cholera. Friedberger and Moreschi and other authors are of the opinion that the partial receptors of different typhoid strains vary considerably from one

another and they believe that they have shown that the titer of a bactericidal serum, as determined with one typhoid strain, cannot be considered an absolute measure of its value. That this opinion is incorrect is shown by the results which we have obtained in testing sera 180B and 80B with typhoid strains of different virulence (see tables 4a and 4b).

TABLE 7

Variations in the lethal and L_+ dose of strain Momoyama 2 (effect of animal passage)

DATE OF TEST	LETHAL DOSE	L_+ DOSE
1917	*graduations*	*graduations*
November 1	1.07	1.80
1918		
January 10	1.00	2.00
January 15	1.22	2.60
(passage)		
March 20	0.98	3.90
(passage)		
May 16	0.95	
(passage)		
July 5	1.25	4.30
(passage)		
August 20	1.30	5.00
(passage)		
September 21	1.60	5.60
(passage)		
October 20	1.00	5.50
(passage)		
November 20	0.96	4.90
December 10		5.30
1919		
March 16	1.05	
(passage)		
March 21	0.98	
May 6	0.95	

The results presented in table 4 show that the simultaneous testing of the same serum with different strains of typhoid bacilli leads to the same value. Hence, we must assume that the receptors of different typhoid strains possess no specific differences with respect to the protective antibodies and that each kind of partial antibody corresponds with a common haptophore group of the bacterial protoplasm.

However, in the experiments described above we have shown certain differences in the various typhoid strains; namely, with respect to the L_+ dose. How are these differences to be explained? We must assume with Pfeiffer that the highly complicated molecule of the bacterial protoplasm possesses receptors which seize the corresponding antibodies. In virulent bacteria we must assume an increase in both the number and the avidity of the receptors. The differences in the binding power; that is, the differences in the size of the lethal and L_+ doses are not explicable as due to a dissimilarity of the receptors but by differences in their number and avidity with respect to the antibodies.

If the receptor apparatus of one strain of the bacteria is uniform in it, it should be expected that the L_+ dose, as determined

TABLE 8

Summary of the results of the tests of two antityphoid sera with the three strains of typhoid bacteria

SERUM	WITH TAKAYAMA P	WITH MOMOYAMA 2	WITH STRAIN 637
	units	*units*	*units*
80B	12,000	11,980	12,000
180B	17	18	17

with any standard serum would be available for the testing of all other antityphoid immune sera. That this assumption is not a mistaken one is shown in table 8, which is to be compared with table 5.

It is seen that the values obtained in the two sera with the different strains present no noteworthy differences. The small variations probably represent the inevitable experimental error.

SUMMARY

1. Our experiments have shown that the virulence titer of the living typhoid bacilli is very variable according to the strain and that no quantitative relationship exists between the virulence titer and the binding power with relation to the protective unit of antiserum. For this reason the lethal dose of the bacteria

is not a reliable measure of a protective unit of an antityphoid serum. A reliable test of a bacterial antiserum can be made only through the use of a standard serum.

2. Our new method of testing typhoid antisera is as follows: with the use of an arbitrary protective unit of antiserum (standard serum) the L_+ dose of a typhoid culture is determined; with this L_+ dose the serum to be tested is mixed in varying quantities; the largest amount of the serum which will just permit the death of a guinea-pig of 250 grams weight within twenty-four hours after the mixture has been injected into the peritoneal cavity is taken as the protective unit. The value of an antityphoid serum is expressed by the number of such protective units contained in 1 cc. of the serum.

3. Any strain of typhoid bacteria can be used for the test. The virulence control is not important. It is also unnecessary to attempt to increase the virulence of the cultures. Efforts in this direction have failed in our hands.

4. The general adoption of our method and particularly of our protective unit will naturally permit, for the first time, a quantitative comparison of the antityphoid sera prepared with different strains and in various laboratories.

A NEW METHOD OF TESTING ANTITOXIC DYSENTERY SERUM

YOSHIMOTO FUKUHARA

From the Pathological-Bacteriological Institute in Osaka, Japan
(Director: Professor A. Sata)

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The testing of antitoxic dysentery serum can be carried out by injecting mixtures of the toxin and antitoxin and also by injecting these two reagents separately. Kraus and Doerr made use of the latter method, while Todd, Villiard and Dopter, Kolle and his co-workers, Schottelius and also Inomata used the former method with sufficiently reliable results.

Kraus and Doerr carried out the test of the serum upon rabbits of 800 to 1000 grams weight. They injected four lethal doses of the toxin into the marginal vein of one ear and, at the same time, varying quantities of the serum to be tested into the marginal vein of the opposite ear. On the basis of their experiments these authors thought that only such sera should be used in the treatment of human dysentery that could protect in a quantity of 0.1 cc.

However, according to our experiences the separate injection method produces variable results and it is, therefore, unreliable.

Inomata, working in the sero-therapeutic institute in Osaka, recommended the following mixture method. Thirty lethal doses of the fluid dysentery toxin were mixed with diminishing amounts of serum and after being kept an hour at 37°C. the mixtures were injected intravenously into rabbits. The protective unit of the serum was taken as the smallest amount which would just prevent the death of the animal. My own comparative experiments have convinced me, also, that the determination of the immunizing and curative value of anti-dysenteric sera is best accomplished with the mixture method.

An inevitable requirement of a method of testing the antitoxic power of a serum is the establishing of a uniform standard of measurement. The previous authors have used the lethal dose of the toxin as the toxic unit. However, the fluid dysentery toxin loses its toxicity gradually, even when it is carefully protected against injurious influences. Furthermore, it was not known whether the toxicity and binding power of dysentery toxin are parallel functions. However, we have found that dysentery toxin can lose its toxicity while retaining its binding power. This instability of the fluid dysentery toxin led us to adopt, as a standard of measurement, an arbitrary unit of the stable antitoxin as was done by Ehrlich in the testing of diphtheria antitoxin. This necessitated the preparation of a "standard serum," which should be used not only in the testing of the new antitoxic sera but also in the study of the constitution of the dysentery toxin itself.

PREPARATION OF THE DYSENTERY TOXIN

Not every dysentery strain is suitable for the preparation of soluble toxin. We have examined a series of 10 strains with respect to toxin production and among these we have found only one satisfactory toxin producer (strain Fujimoto) for which I am indebted to Dr. Inomata. As a culture medium we have used a mixture of a solution of pepton prepared from pigs' stomachs with meat infusion. The meat infusion was prepared by boiling the chopped meat with acetic acid according to Hida, in order to destroy the muscle sugar. The extract was obtained, as usual, by filtration. A reaction of 5 per cent of normal NaOh beyond the neutral point with phenolphthalein gave the best results. The strongest toxin that we obtained killed 1500 gram rabbits within five days after the intravenous injection of 0.01 cc. or occasionally as little as 0.005 cc. The first toxin used in our experiments was obtained by filtration of the three weeks broth culture. The clear fluid thus obtained was preserved with toluol. The direct toxicity was measured by the amount that could kill a rabbit of 1500 grams weight by intravenous injection in four

or five days. Larger animals require correspondingly larger amounts of the toxin. Smaller amounts than the selected lethal dose may cause the death of the animals in from eight to eleven days or they may fail to kill but produce paralysis. The use of a number of animals is advisable as this permits a more exact estimation of the toxicity. A similar advantage is secured by the use of larger series of animals in the testing of the antitoxic sera. We shall revert to this point later.

THE SELECTION OF THE STANDARD UNIT OF ANTITOXIN AND THE CORRESPONDING TOXIN UNITS; NAMELY, THE L_0 AND THE L_+ DOSES

The standard antitoxin unit which we adopted was, naturally, an arbitrary one. We took it as the smallest amount of the serum which could just prevent the death of the animal when injected intravenously in combination with 100 minimal lethal doses (2 cc.) of the toxin that happened to be at our disposal. The protocol of the experiment in which this quantity was arrived at is shown in table 1.

From the results of this experiment it was calculated that 1 gram of the dried serum contained 42 antitoxin units.

The determination of the L_0 and L_+ dose had, naturally, to be carried out with the use of the above selected standard serum. The interval between these two doses is very variable. By repeated tests with the L_0 dose we have found that this unit of the toxin is not practically useful in the testing of antitoxic sera. The L_+ dose was, therefore, preferred for that purpose. The criterion in the use of the latter unit is the death of a rabbit of 1500 to 2000 grams weight within four to five days. In all of these tests, whether for the determination of the L_+ dose or for the testing of an antitoxic serum, it is advisable to employ a number of animals in order to avoid the difficulty of individual variations in the resistance of the animals.

If an antidysentery serum is to be tested as to its antitoxic value the L_+ dose of the toxin that is to be used in the test must first be determined. Table 2 presents some examples of tests conducted to this end.

TABLE 1

The testing of the original "standard serum"

SERUM DILUTION	WEIGHT	RESULTS
	grams	
1:140	1100	Paralysis; dead after 2 days
1:100	1200	Dead after 1½ days
1:80	1320	Lived
	1780	Paralysis; dead after 2½ days
1:75	1870	Paralysis; dead after 2½ days
	1200	Paralysis; dead after 7 days
1:70	2100	Lived
	1350	Dead after 9 days
1:65	1730	Lived
	1350	Paralysis; dead after 6 days
1:60	1350	Paralysis; recovered
	1640	Lived
1:55	1230	Lived
	1620	Paralysis; dead after 4 days
1:50	1400	Lived
	1400	Paralysis; dead after 16 days
1:45	1580	Dead after 2½ days
	1680	Paralysis; dead after 9 days
1:43	1580	Paralysis; dead after 3 days
1:42	1470	Lived
	1270	
1:41	1610	Lived
1:40	1700	Lived
	1400	Lived
1:35	1500	Lived
	1440	Dead after 13 days

TABLE 2

Determination of the L_0 and L_+ dose of dysentery toxin 9

ONE UNIT OF ANTITOXIN PLUS THE AMOUNTS OF TOXIN MENTIONED BELOW	RESULTS
cc.	
0.8	Lived
	Lived
0.9	Lived
	Lived
1.0	Lived
	Lived
1.1	Paralysis; recovered
	Lived
1.4	Dead after 2½ days
	Paralysis; recovered
1.5	Lived
1.7	Lived
	Dead after 2½ days
1.8	Paralysis; recovered
	Paralysis; recovered
1.9	Paralysis; recovered
	Paralysis; dead after 5 days
2.0	Paralysis; recovered
	Dead after 2 days
2.1	Paralysis; recovered
	Paralysis; dead after 2½ days
2.2	Paralysis; dead after 7 days
	Paralysis; dead after 1½ days
2.3	Paralysis; recovered
	Dead after 1½ days
2.4	Paralysis; dead after 1½ days
	Paralysis; dead after 3 days

It is seen, here, that the injection of one antitoxin unit mixed with 1 cc. of toxin caused symptoms but no paralysis. With 1.01 cc. of the toxin, paralysis resulted; consequently, the L_0 dose was taken as 1 cc. With 2.1 cc., 2.2 cc. and 2.3 cc. the toxic

TABLE 3

The testing of anti-dysentery serum 237 with dried dysentery toxin B (L_+ dose 0.39)

SERUM DILUTED	WEIGHT	RESULTS
	grams	
1:125	1550	Lived
1:150	1720	Lived
1:175	1600	Lived
1:200	1470	Lived
1:225	1440	Paralysis; dead after 2½ days
	1540	Lived
1:260	1390	Lived
	1350	Dead after 2½ days
1:275	1720	Paralysis; dead after 5 days
	1430	Lived
1:290	1810	Lived
	1790	Dead after 3½ days
1:300	1560	Dead after 8½ days
	1850	Paralysis; recovered
1:305	2020	Dead after 1½ days
	1750	Paralysis; dead after 3½ days
1:310	1290	Paralysis; dead after 3 days
	1780	Dead after 3 days

effect becomes greater. Finally, all the animals that received mixtures containing 2.4 cc. regularly die after two to three days. This amount, therefore, was taken as the L_+ dose.

The fluid toxin preserved under toluol becomes constantly weaker. For this reason the fluid toxin must be tested from time

to time with the standard serum with respect to the L_+ dose. The minimal lethal dose of the toxin must, also, be determined. If it is desired to guarantee the constancy of the L_+ dose in order to avoid its repeated determination, we must use a dry toxin obtained by precipitation with ammonium sulphate. Such dried toxin preserved in Ehrlich vacuum tubes can maintain its properties for years.

The testing of antidysentery serum is conducted with the use of the L_+ dose of the toxin in the manner similar to the method of testing diphtheria antitoxin. The mixtures, after being kept for an hour at 37°C. are injected intravenously into rabbits of 1500 to 2000 grams weight. The largest amount of the serum which when mixed with the L_+ dose of toxin and injected intravenously will permit the death of the animal in four to five days is taken as the antitoxic unit for that serum. The results of such a test are shown in table 3.

It is seen that in this instance 1 cc. of the serum 237 contains 305 antitoxin units.

Our standard serum is preserved, as usual, in the dry state in vacuum tubes. Our institute will be glad to supply tested standard serum upon request.

THE BINDING RELATION OF THE DYSENTERIC TOXIN AND ANTITOXIN

According to Todd, the union between dysentery toxin and its antitoxin takes place considerably more quickly at warmer temperatures than it does in the cold. At 37°C. five minutes were sufficient, whereas two hours were required for complete neutralization at 0°C. Our experiments, also, indicate that differences in temperature exert a noteworthy influence on the course of the toxin-antitoxin reaction. The protocol presented in table 4 shows that complete union takes place after one hour at body temperature. The union thus takes place rather slowly. However, one can be sure of complete union within one hour at 37°C.

If the dysentery toxin behaved like diphtheria toxin, it should be expected that the minimal lethal dose would increase with time, while the binding power would remain unchanged; that

is, the L_+ dose would not increase. However, the results of tests presented in table 5 show that this is not the case.

TABLE 4

Showing the influence of time on the reaction between dysentery toxin and antitoxin

TOXIN	INJECTED AFTER BEING MIXED WITH ONE UNIT OF THE ANTITOXIN			
	At once	After 30 minutes at 37°C.	After one hour at 37°C.	After one hour at 40°C. and 20 hours at 20°C.
cc.				
1.5	Lived			
1.7	Lived		D, 2½	
1.8	Par., Rec.		Par., Rec.	
1.9	Par., D 4	Lived	D, 9½ Par., D 2½	Par., Rec. Par., D 5.
2.0	Par., D 2	Lived	D 1½ Lived	D 2 Par., Rec.
2.1	Par., Rec.	Par. D 5½ D 2	Par., D 2½ Par., Rec.	Par., D 8½ Par., D 1½
2.2	Par., Rec. D 20 hours	Lived Lived	Par., D 1½ Par., D 7	Par., D 5½ Par., Rec.
2.3	D 1½	Par., D 2½ D 2	D 1½ Par., Rec.	Par., Rec. Par., D 5
2.4	D 1½		Par., D 2½ Par., D 3	Par., D 1½ Par., D 3
2.5	Par., D 2½		D 1½	D 4 D 2
2.6				D 1½ D 1½

Par. = paralysis; Rec. = recovered; D = died (the numerals indicate the number of days that the animal survived the injection).

It is seen that in this case as the lethal dose increased from 0.009 cc. to 0.08 cc. the L_+ dose rose from 1 cc. to 7.5 cc. This experiment makes it probable that the changes in the dysentery

toxin represents an alteration into non-toxic substances which possess no avidity for the antitoxin. Expressed in the terminology of Ehrlich, a destruction of the toxin had taken place which affected not only the so-called toxophore group but also the haptophore group. The changes are, thus, different from those which take place in diphtheria toxin.

TABLE 5

Influence of age on the toxicity and the binding power of dysentery toxin 9

DATE OF TEST	MINIMAL LETHAL DOSE	L_+ DOSE
August 28, 1916	0.009	1.00
October 18, 1916	0.012	1.20
November 9, 1916	0.014	1.23
November 14, 1917	0.080	7.50(?)
February 5, 1918	0.170	*

* The L_+ dose, at this date, was too large to be estimated.

SUMMARY

1. In order to determine the antitoxic value of anti-dysentery serum it is necessary to select a standard serum which can be preserved in a dry state in the vacuum tubes of Ehrlich.

2. The standard antitoxin unit adopted by us was the amount that neutralized 100 minimal lethal doses of the toxin which at that time was at our disposal.

3. For the testing of other anti-dysentery sera the so-called L_+ dose of dysentery toxin was adopted as the smallest amount which when mixed with the antitoxin unit and injected intravenously into a rabbit of 1500 to 2000 grams weight caused the death of the animal within from four to five days. The L_+ dose of the toxin is employed, in the usual manner, to determine the relative antitoxic value of newly prepared antisera.

4. The deterioration of aged dysentery toxin is referable to a change of the toxin molecule into a non-toxic modification which possesses no avidity for the antitoxin. The toxin molecules lose their toxicity without the formation of toxoids; in this repect the dysentery toxin is unlike diphtheria toxin.

EXPERIMENTAL PURPURA

MARK J. GOTTLIEB

From the Laboratory for Clinical Research, New York, N. Y. Contribution No. 1

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Although purpura has been known for a long time as a disease in which hemorrhages occur spontaneously or from slight provocations, it has only been of late that we have recognized the fact that a condition may exist in the individual wherein after the tissues have been cut or bruised, they will bleed for an abnormally long time. Neither the person so afflicted nor his medical attendant is cognizant of his condition because these patients do not show outward signs of the disease except after sustaining an accidental or surgical wound. Associated with this prolonged bleeding is a diminution in the number of blood platelets and the red blood cells are easily laked by hypotonic or hypertonic salt solutions.

Hess (1), who has studied this condition carefully, suggests that in the blood stream of these individuals there exists a toxic substance which has the power of dissolving the blood platelets and of rendering the erythrocytes easily laked by heterotonic salt solution and that this substance causes a disturbance in the nutrition of the lining of the blood vessels. He believes that the reduction in the number of the blood platelets is more apparent than real and comes to this conclusion after an experimentation by which he finds that although the number of visible blood platelets be reduced, the remaining portion exists in solution in the blood stream.

Lee and Robertson (2) have been able to produce experimental purpura in guinea-pigs. They proceeded as follows.

The washed blood platelets of two guinea-pigs were injected intravenously into a rabbit and this was repeated every seven days for four

successive times; on the seventh to the tenth day after the last administration of guinea-pigs' blood platelets, either the rabbit was bled to death or blood was withdrawn from the heart and the serum thereof was injected intraperitoneally into guinea-pigs in varying doses; as a result of this procedure a condition was produced in these animals wherein the bleeding time was very much prolonged, while the coagulation time remained normal; the blood platelets were reduced in number. Those animals which received a sufficiently large dose of the serum usually died and on post-mortem examination there were found blood clots and blood stained fluid in the pleural and peritoneal cavities, hemorrhages into the walls of the intestines, heart muscle and liver. Before death there was a continuous ooze of blood from small cuts which were made in the ears for the purpose of procuring blood for the various tests. This anti-platelet serum not only had the property of producing this condition in the living animal but it also dissolved blood platelets *in vitro*.

We have repeated this experiment in the following way: one or two guinea-pigs, depending upon size, were exsanguinated by severing the vessels of the neck and allowing the blood to flow into a flask containing citrate solution; the blood thus obtained was centrifuged at high speed for fifteen minutes, and after the supernatant fluid was removed, the white coat which was found on the surface of the sediment was pipetted off; normal saline solution was added to the collected blood platelets and the mixture was thoroughly agitated; this was centrifuged again for fifteen minutes; the supernatant fluid was removed and the layer of blood platelets was pipetted off and again washed as before; the washed platelets were then suspended in saline solution and injected intravenously into a rabbit; although we have washed blood platelets many times, we have never been able to procure them entirely free from red blood cells, probably because the quantity of blood used was small and in our endeavor to procure the maximum amount of platelets some red cells were naturally taken up with them; the rabbits were subjected to weekly intravenous injections of the blood platelets thus procured according to the succeeding tabulation.

White rabbit no. 1; weight, 2006 grams

December 30, 1918. Blood platelets of 1 large guinea-pig, intravenously.

January 6, 1919. Blood platelets of 2 large guinea-pigs, intravenously.

January 14, 1919. Blood platelets of 2 medium sized guinea-pigs, intravenously.

January 21, 1919. Blood platelets of 1 large guinea-pig, intravenously.

January 31, 1919. Blood platelets of 2 small guinea-pigs, intravenously.

February 13, 1919. Etherized and exsanguinated and the blood collected under as steril conditions as possible.

TIME	ANGORA GUINEA-PIG, 501 GRAMS	BLACK GUINEA-PIG, 490 GRAMS
February 11, 1919:		
Coagulation time	7 minutes	5 minutes
Bleeding time ...	9 minutes	9 minutes
Blood platelets..	484,000 per cubic centimeter	649,000 per cubic centimeter
6 p.m.....	2 cc. normal rabbit's serum given intraperitoneally	1.6 cc. serum from rabbit number 1 (anti-platelet) intraperitoneally
February 12, 1919, 10 a.m:		
Coagulation time	6 minutes	6 minutes
Bleeding time....	10 minutes	Continuous
Blood platelets...	250,000	333,000
Urine.....	Normal	Bloody and contained moderate number of red blood cells and a few hyalin casts.
February 12, 1919, 4.30 p.m:		
Coagulation time	3 minutes	2 minutes
Bleeding time ...	10 minutes	Continuously bleeding
Blood platelets ..	435,000	171,000
February 13, 1919; 9 a.m.	Alive and well	Found dead.*

* *Autopsy notes.* Bloody discoloration of peritoneum. Small quantity of blood stained fluid and clots in peritoneal cavity. Hemorrhagic area in the wall of the right auricle. Blood stained fluid in pleural cavity. Right lung intensely congested but not consolidated.

Two guinea-pigs of approximately equal weight were selected and action of the serum obtained from white rabbit number 1 was compared with the action of normal rabbit's serum.

It will be noticed in the above protocol that 2 cc. of normal rabbit serum were given to the angora pig while 1.6 cc. of anti-platelet serum were given to the black pig. There was a temporary reduction of blood platelets in the blood of the pig that received normal rabbit serum while the reduction in the number of blood platelets in the pig that received anti-platelet serum was progressive.

The activity of the serum of rabbit number 1 (anti-platelet) against guinea-pig's blood platelets as compared with the action of normal rabbit serum was also determined. In this connection it might be well to add that Lee and Robertson found that complement is essential in the reaction and we accordingly used the rabbit serum on the same day that it was obtained, in order that the complement that is normally present in sera would be operative in the tests without the addition of extraneous complement.

In the following protocol 0.5 cc. of diluted serum was mixed with 0.05 cc. of a 5 per cent suspension of guinea-pig's blood platelets in 0.9 per cent salt solution. After the mixtures were made, they were incubated for one hour in the water bath at 37°C. and the results were then read.

DILUTION	RABBIT 1	NORMAL RABBIT
1–1	Complete lysis	Complete lysis
1–2	Complete lysis	Complete lysis
1–4	Complete lysis	Complete lysis
1–6	Complete lysls	Complete lysis
1–8	Complete lysis	Partial lysis
1–10	Complete lysis	No lysis
1–12	Complete lysis	No lysis
1–15	Complete lysis	No lysis
1–20	Complete lysis	No lysis
1–25	Complete lysis	No lysis
1–30	Complete lysis	No lysis
1–40	Complete lysis	No lysis
1–50	Complete lysis	No lysis
1–60	Complete lysis	No lysis
1–80	Partial lysis	No lysis

From the foregoing we learn that normal rabbit serum is lytic to guinea-pigs' blood platelets in dilutions of the serum not higher than one to eight, whereas the serum of the rabbit that was treated with guinea-pig's blood platelets dissolved them in dilutions far greater.

Because of the fact that we were never able to obtain blood platelets entirely free from red blood cells, it is quite natural that we should endeavor to determine how much hemolytic antibody was produced in the rabbit in conjunction with the platelet anti-body (3). A rabbit was prepared as in the case of rabbit 1 and its serum was used on the same day that the blood was removed from its heart. This serum was used in the following experiment.

In a set of test tubes 0.5 cc. of diluted serum and 0.05 cc. of a 5 per cent suspension of guinea pigs' red blood cells were mixed. In another set 0.5 cc. of diluted serum and 0.05 cc. of a 5 per cent suspension of guinea-pigs' blood platelets were mixed. The tests were incubated for one hour at 37°C. and then read.

Rabbit serum 2

DILUTION	PLATELETS	ERYTHROCYTES
1–6	Complete lysis	Complete lysis
1–8	Complete lysis	Complete lysis
1–12	Complete lysis	Complete lysis
1–16	Complete lysis	Complete lysis
1–24	Complete lysis	Complete lysis
1–32	Complete lysis	Partial lysis
1–48	Complete lysis	Partial lysis
1–64	Complete lysis	Partial lysis
1–96	Partial lysis	No lysis
1–128	Nearly complete lysis	No lysis
1–192	No lysis	No lysis
1–256	No lysis	No lysis
1–384	No lysis	No lysls

Here we find that a hemolytic antibody was produced in conjunction with the platelet antibody but, apparently, not in as great an amount. The question must necessarily arise in one's mind: is this hemolytic antibody also a factor in the production

of so-called experimental purpura or does it exert the preponderating influence in its production? This matter will be reported in a succeeding contribution (4).

Black rabbit 3 was injected intraperitoneally with the blood serum of rabbit 2 for the purpose of determining whether the purpuric serum of one rabbit would produce in a normal rabbit protective substances against the action of this purpuric serum or whether the transferred purpuric serum would confer upon the serum of the recipient rabbit the property of producing purpuric symptoms. The following protocol indicates how the serum was administered.

April 25, 1919. 2 cc. of rabbit serum 2 injected intraperitoneally into rabbit 3.

April 26, 1919. 2 cc. of rabbit serum 2 injected intraperitoneally into rabbit 3.

April 28, 1919. 2 cc. of rabbit serum 2 injected intraperitoneally into rabbit 3.

April 30, 1919. 2 cc. of rabbit serum 2 injected intraperitoneally into rabbit 3.

May 2, 1919. 2 cc. of rabbit serum 2 injected intraperitoneally into rabbit 3.

May 5, 1919. 2 cc. of rabbit serum 2 injected intraperitoneally into rabbit 3.

May 7, 1919. 2 cc. of rabbit serum 2 injected intraperitoneally into rabbit 3.

May 9, 1919. 2 cc. of rabbit serum 2 injected intraperitoneally into rabbit 3.

May 19, 1919. Paracentesis cardia was performed on rabbit 3 and the serum obtained was used in the following experiment.

The lethal dose of the serum of rabbit 2 (anti-platelet) was determined to be about 1.2 cc. This dose was sufficient to cause the death of guinea-pigs whose average weight was 500 grams, within forty-eight hours. The dose used in this last experiment was 1.5 cc.

	GUINEA-PIG		
	No. 1	No. 2	No. 3
Weight.....	473 grams	478 grams	463 grams
May 21, 1919:			
Bleeding time.	15 minutes	15 minutes	33 minutes
Platelets.....	281,000	266,000	510,000
5.12 p.m.....	1.5 cc. serum rabbit 2 (antiplatelet) intraperitoneally	1.5 cc. serum rabbit 2 (antiplatelet) intraperitoneally	1.5 cc. serum rabbit2 (antiplatelet) intraperitoneally
May 22, 1919:			
10.30 a.m.			
Bleeding time.	1 hour 5 minutes	Continuous bleeding	Continuous bleeding
Platelets.....	77,000	57,000	128,000
10.45 a.m.		2 cc. serum rabbits 3 intraperitoneally	2 cc. normal rabbit serum intraperitoneally
4.20 p.m.....			
Platelets.....	52,000	278,000	325,000
Bleeding time.	Bleeding continuously	Bleeding continuously	Bleeding continuously
5 p.m.....		2 cc. serum rabbit 3 subcutaneously	2 cc. normal rabbit serum subcutaneously
May 23, 1919:			
9 a.m.....	Found dead	Still alive	Still alive
May 24, 1919:			
3 p.m.....		Found dead	Still alive
Autopsy.....	Hemorrhages into subcutaneous tissues, intestines, stomach, and heart muscle	Hemorrhages into intestines, stomach, liver and heart	

It will be noticed in the above cited experiment that in the case of guinea-pig 3 normal rabbit's serum apparently had the effect of counteracting the action of the anti-platelet serum. The serum of rabbit 3 did not seem to be as effective as normal rabbit's serum; the life of guinea-pig 2 was prolonged but its death was not prevented. In this instance we may infer that the treatment of rabbit 3 with the serum of rabbit 2, partly nullified the protective qualities of the serum of rabbit 3.

Whether the serum of rabbit 2 remained operative as such in rabbit 3 or whether it produced some substance in rabbit 3 which offset those protective agents, remains open for further investigation.

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THE ANTIGENIC PROPERTY OF THE PFEIFFER BACILLUS AS RELATED TO ITS VALUE IN THE PROPHYLAXIS OF EPIDEMIC INFLUENZA

CHARLES W. DUVAL AND WILLIAM H HARRIS

From the Department of Pathology and Bacteriology, Tulane University of Louisiana

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During the height of the epidemic of influenza which occurred in New Orleans in the fall of 1918, when the Health authorities were recording between two and three thousand new cases daily, we undertook to determine the value, if any, of specific bacterial protein in the prevention of the infection. Prior to the systematic vaccination of a relatively large number of persons, we had satisfied ourselves (1) that the Pfeiffer bacillus played an important rôle in the clinical disease known as epidemic influenza. Furthermore, while this work was in progress we noted that *B. influenzae* possessed distinct antigenic properties, which indicated that some degree of protection against the disease might be afforded in man through its employment as a vaccin.

The futility of all other methods to check the overwhelming spread of influenza suggested efforts along prophylactic lines already established and well recognized for various other epidemic diseases. Inasmuch as previous visitations of epidemic influenza extended over a period of only a few weeks, the possibility of establishing protective sensibilizators sufficient for such a duration, seemed to justify attempts at transient immunization with specific bacterial proteins. Again, if the epidemic continued in violence for a longer period revaccination could be carried out. With these basic principles at hand, we instituted to as great an extent as possible this method of defense after proper representation to those concerned of its hypothetical limitations. We further realized that at the time of the previous great epidemic in 1890–92 the influenza bacillus was discovered late in the epidemic

period (2) and bacterial vaccin immunization had not yet formed a part of our protective armamentarium. Therefore, it was apparent that only a thorough trial of vaccin in the present epidemic could demonstrate its degree of efficiency or possible worthlessness; in either instance no harm could result in consequence of its administration. The successful accomplishment with other vaccins such as small-pox, typhoid, and more recently the meningococcus, encouraged the hope that perhaps at least something might be achieved in the use of a vaccin of the Pfeiffer bacillus or that knowledge bearing upon its status as a preventive method might be gained for consideration of its employment in future epidemics.

Vaccin and its preparation. As it was our express purpose to attempt the protection against a specific infection caused by one and not several bacterial agents, we employed as a vaccin the protein of killed cultures of *B. influenzae.* We did not consider that there was anything to be gained for prophylaxis against Pfeiffer infection through the use of mixed vaccin (3), or one that contained, with *B. influenzae,* the pneumococcus, streptococcus, staphylococcus, etc., since these latter species, in our opinion, play only a secondary rôle in the epidemic disease. While the use of a heterologous vaccin could do no harm there seemed no occasion to attempt the immunization against possible secondary invaders, particularly as their activity followed the infection of the host by some other excitant. Even though the disease is not caused alone by the Pfeiffer bacillus, it was considered logical to attempt the prevention of the infection by exciting, in the prospective host, immune substances specific for this microorganism. If the primary infection could be prevented, dependent secondary infections in consequence would not arise. To accomplish this protection the Pfeiffer antigen alone was employed to stimulate the maximum amount of antibody production without simultaneous interference with the mechanism by other antigens.

The cultures included several isolations from recent cases of the epidemic disease and a strain of *B. influenzae* (Wollstein) obtained from the Rockefeller Institute which has been in

our possession for a number of years. While at first it was thought advisable to use a number of influenzal isolations, having in mind the possible existence of types it was later found unnecessary as the agglutination tests carried out upon patients' sera did not suggest "variants" of *B. influenzae*, the absence of which seemed already determined by Wollstein (4) in her study of a large number of isolations from widely separated localities and from various pathological conditions.

In regard to the antigenic property of various influenzal cultures it was found that the one from the Rockefeller Institute possessed this to a high degree, and perhaps greater than other cultures employed by us. Because of its power constantly to produce specific antibodies and to give rise to a marked local and constitutional reaction in the vaccinated host this culture was finally employed to the exclusion of all others.

The influenza bacilli for vaccin were grown upon the surface of a solid nutrient agar medium, 0.6 per cent acid against phenolphthalein, to which was added before allowing to solidify, one per cent steril, saline washed human erythrocytes (complement free). These were thoroughly mixed with the liquid agar medium in large Erlenmeyer flasks and the admixture was cooled rapidly to ensure against settling out of the erythrocytes away from the surface on which growth was to be encouraged. Where freshly drawn blood was used, the defibrinated sero-cellular part was heated at 56°C. to destroy the complement before adding it to the nutrient medium. The solidified culture medium was seeded by flooding the surface with a fresh saline suspension of *B. influenzae*, introduced by means of a sterile pipet. Incubation at 37°C. was carried over a period of 48 hours, and the growth was then washed off with steril saline solution; care was taken not to disturb the culture medium. The collected suspension was now devitalized by saturation with chemically pure chloroform (Merck's) which was allowed to remain for ten to fifteen minutes in the admixture. The mixture was agitated from time to time to insure uniform diffusion of the germicidal agent. Repeated tests showed that the chloroform killed *B. influenzae* in a few seconds, and apparently without injury to the antigenic

property of the bacterial protein even though this chemical remained in contact with the culture for a period of days. After the culture was devitalized the chloroform was allowed to settle out which in greater part occurred within a few minutes. The supernatant suspension of killed bacilli was now removed to steril flasks. These were lightly plugged with cotton and placed at 37°C. for a time sufficient to remove all traces of chloroform, which usually required less than one hour. The chloroform-free culture was then standardized and tested for viability after which it was ready for use. The vaccin prepared in this manner gives a suspension of bacterial protein free from all other extraneous irritants and unappreciably altered in its antigenic power.

For inoculation purposes we employed only the freshly prepared vaccin (not more than a week old). The vaccinations were all made by subcutaneous injection into the outer aspect of the arm below the insertion of the deltoid muscle. The dose employed for adults was approximately one billion killed *B. influenzae* for the first injection, one-half this number for the second, and one billion for the third injection, allowing an interval of three days between the inoculations. Arbitrarily it was decided that three doses constituted a complete treatment, following in this respect the ordinary method of vaccination against typhoid fever. We did not consider lipovaccin under the existing conditions of the epidemic for the reason that its action might be slower, and we desired the maximum amount of host response in the shortest possible period.

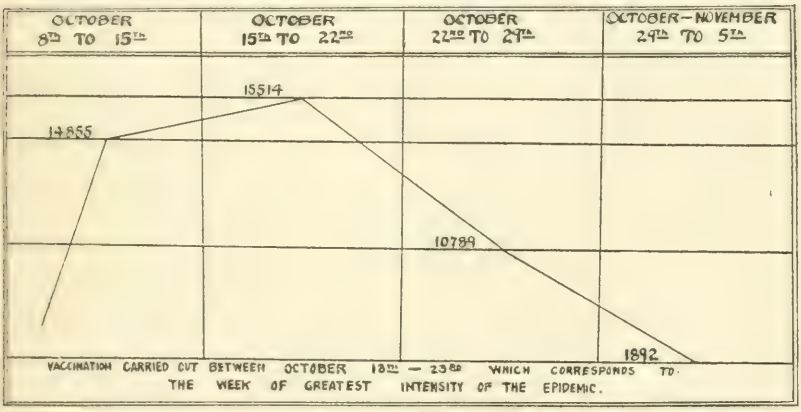
OBSERVATIONS UPON THE EFFECTS OF VACCINATION

Approximately five thousand persons were vaccinated by subcutaneous injection with influenzal protein which had been specially prepared by devitalizing the culture with chloroform. The majority of those vaccinated were employees in the large commercial houses, banks and factories of the City of New Orleans. Because of the interest and cooperation taken by the various heads of these establishments it was possible systemati-

cally to observe the effects of the vaccin upon selected groups for a period of months after the injections. In addition to these groups we vaccinated several thousand persons widely distributed over the city. In each instance the vaccin was administered to those who at the time were apparently well, and stated that, so far, they had escaped influenza infection.

For our group series the vaccinations were completed within a period of two weeks during which time the epidemic had reached its maximum intensity (see chart). However, before the epidemic showed any signs of recession our series in greater part had received the complete vaccin treatment, namely three doses given at intervals of three days between each injection.

CHART— SHOWING NUMBER OF CASES PER WEEK FOR CITY OF NEW ORLEANS.



Eight weeks after the subsidence of the epidemic, a second visitation (recrudescence) occurred, which afforded an unusual opportunity to determine in those previously vaccinated the duration of protection occasioned by the specific protein of *B. influenzae*.

Out of a total number of our group series, 2608 persons who received the complete treatment of vaccin (three injections) 98.3 per cent did not contract the disease. Of the total number of 346 who received only two injections 92 per cent did not contract influenza during the first epidemic. Seventy-six per cent of the 118 receiving but one injection did not develop the

disease. This latter group shows a marked difference in the percentage of infection compared with those receiving the full treatment. It is noteworthy that in those vaccinated that developed influenza the character of the infection was mild and without pneumonic complications. There were 866 individuals, forming a part of the forces vaccinated, who refused injection; 375 of these or 41 per cent developed influenza as contrasted with only 3.3 per cent occurrence of the disease in those vaccinated. These persons afforded a means of control inasmuch as their occupation and daily environment were identical with those that were vaccinated. While the incidence of infection is higher in the controls than that shown for the general city statistics, the difference is explained by the fact that these individuals were employed in large establishments located in the congested business sections.

Tables 1 and 2 indicate the results with individual vaccinated groups. Table 3 presents a general summary with controls of the results obtained in all groups.

While we realize that the number of persons vaccinated is too small to draw any sweeping conclusions relative to the percentage of absolute protection afforded and the duration of the immunity we believe, however, that even in these relatively few cases, the results indicate specificity of the Pfeiffer bacillus and efficacy of the vaccin as a prophylaxis in the infection.

The clinical reaction. In the majority of cases a reaction occurred at the site of inoculation, usually in the form of a localized erythema. This was anything from a mild circumscribed redness, 4 to 5 cm. in diameter, to a markedly swollen and reddened skin and subcutaneous tissue involving the whole arm and the greater part of the forearm. This local inflammatory reaction gradually subsided and the redness faded out in three to five days. In addition to the local effects there occurred, in the majority of the cases, a well defined constitutional reaction. This host response was frequently so striking in many of its clinical aspects as to simulate the early stages of influenza. In some instances the reaction was severe enough to confine the individual to bed for a period of eight to ten hours, such usually

TABLE 1

Showing results of vaccination for individual group A

TOTAL NUMBER EMPLOYEES	NUMBER SICK AT TIME	NUMBER VACCINATED		NUMBER REFUSING VACCINATION	
1000 (100%)	364 (36.4%)	398 (39.8%)		238 (23.8%)	
		Number developing influenza	Number not developing disease	Number developing influenza	Number not developing disease
		5 (1.2%) No pneumonia	393 (98.8%)	93 (39%) 27 pneumonia	145 (61%)

	TOTAL NUMBER VACCINATED PERSONS, GROUP A	DEVELOPED INFLUENZA		
		Number of persons	Per cent	Protected
				per cent
One injection	26	4	15	85
Two injections	30	1	3.3	96.7
Three injections	342	0	None	100

TABLE 2

Showing results of vaccination for individual group B

TOTAL NUMBER EMPLOYEES	NUMBER SICK AT TIME	NUMBER VACCINATED		NUMBER REFUSING VACCINATION	
1200 (100%)	517 (43%)	583 (48%)		100 (8%)	
		Number developing influenza	Number not developing disease	Number developing influenza	Number not developing disease
		22 No pneumonia	561	37 14 pneumonia	63

	TOTAL NUMBER VACCINATED PERSONS, GROUP B	DEVELOPED INFLUENZA		
		Number of persons	Per cent	Protected
				per cent
One injection	40	14	35	65
Two injections	191	8	41.8	58.2
Three injections	452	0	None	100

appearing as early as six to eight hours after the first injection of the vaccin. We wish to mention in this connection that the severity of the reaction accounted for a number of our cases receiving only one injection, the individuals stating they did not care to submit to the inconvenience of a second inoculation. In our experience the second injection of vaccin rarely gave rise to more than a mild constitutional reaction, and the third to little if any at all.

In general some form of constitutional response to the vaccin

TABLE 3

Summary of the results of collective groups

	NUMBER OF PERSONS VACCINATED	VACCINATED DEVELOPING INFLUENZA			CONSTITUTIONAL REACTION		
		Number	Per cent	Protected	Severe	Mild	None
				per cent	*per cent*	*per cent*	*per cent*
One injection	118	29	24	76			
Two injections	346	28	8	92	30	60	10
Three injections	2608	45	1.7	98.3			
Total	3072	102	3.3	96.7			
		CONTROLS DEVELOPING INFLUENZA			INCIDENCE OF DISEASE		
		Number	Per cent	Protected	Vaccinated	Unvaccinated	Difference
				per cent	*per cent*	*per cent*	*per cent*
Controls	866						
Persons not vaccinated		375	41.6	58.4	3.3	41.6	38.3

occurred in 90 per cent of the cases. The severe reaction was noted in 30 per cent. Only in 10 per cent did an appreciable reaction fail to develop (see table 3).

In persons reacting the constitutional symptoms ranged from a slight headache, mild pains over the body, lassitude and a half to one degree of temperature, to severe frontal and occipital headache, neuralgic pains over the body, not infrequently ushered in with chill and nausea and followed by a temperature of 101° to 102°. It is noteworthy that even with persons that responded more violently to the vaccin, the reaction invariably subsided

in from six to eight hours after the onset, leaving the individual perfectly normal to all intent and purpose.

As the epidemic was raging at the time vaccination was instituted we expected to have a number of cases develop the disease shortly following the first injection of vaccin. We reasoned that a number, though apparently well at the time, would in reality be in the later stage of the incubation period, and in consequence not to be benefited by vaccin therapy. Under these circumstances it was surprising how few developed the infection during the process of the vaccination period. However, our tabulated results do not exclude any case that contracted influenza after the first injection of vaccin. Such results would indicate that the negative phase, considered by some as a contraindication for vaccination during the epidemic, does not exist or is a negligible factor.

Blood findings. With a view of determining whether the leucocytic blood picture is altered or influenced by vaccin injection and to compare it with that noted in influenza infection, total and differential counts were made upon selected groups of cases before and after vaccin administration. In the vaccinated instances the leucocytic count was, as a rule, somewhat higher than normal, the rise being due to the neutrophiles (5). Leucopenia was not noted in any of the vaccinated persons, but on the contrary the leucocytic count ranged from 9000 to 12,000. A diminution in the normal number of leucocytes which is a characteristic feature in influenza infection was hardly to be expected in the vaccinated case where the antigen is introduced subcutaneously. In this connection it may be mentioned that typhoid antigen injected subcutaneously excites an increase rather than a decrease of the total leucocytic count (6), whereas in the natural disease the organism produces a leucopenia.

Duration of the protection. This could only be approximated by the length of time antibodies for the Pfeiffer antigen remained in demonstrable quantities in the blood of those vaccinated. On the average these substances were found persisting in the sera for a period of two months though often in greatly diminished quantity as compared with that demonstrable ten days to two

weeks after vaccination. However, the second epidemic which occurred about two months after the vaccination, afforded in those again exposed further data bearing upon the duration of the protection. Of those vaccinated at the time of the first visitation of the disease and who successfully passed through this epidemic, 8 per cent contracted influenza during the recrudescence. Of the recorded group controls or those who refused vaccination in October and did not develop the disease during the first epidemic, 56 per cent contracted it in the period of the second epidemic.

TABLE 4

Showing probable duration of protection, group C

PERSONS VACCINATED IN OCTOBER, 1918 (PERIOD OF FIRST EPIDEMIC)	DEVELOPING INFLUENZA IN JANUARY, 1919 (PERIOD OF RECRUDESCENCE)	PERIOD OF PROTECTION
272	21	3 months
Revaccinated in January, 1919	Developing influenza in January, 1919	
154	1	
Total number persons not vaccinated in October, 1919	Developing influenza during first epidemic	Developing influenza during recrudescence
238	93	82

A survey of the results for our series of cases, taking into account only those who did not contract the disease during the second epidemic, indicate two to three months as the average duration of protection afforded by the injection of the chloroform prepared Pfeiffer vaccin.

The antigenic specificity. Inasmuch as the sera from infected individuals with *B. influenzae* yield quite constantly specific agglutinins and to some extent lysins of the complement fixing group, it seemed consistent to expect that normal persons injected with the protein of the Pfeiffer bacillus would likewise contain in the blood specific antibodies. Agglutination and complement fixation tests were therefore carried out upon the sera of a number of those vaccinated in order to obtain an index of the production of such substances. Agglutinins were constantly found present

in variable amounts; the titer, however, was not, as a rule, as great in the sera of the vaccinated individuals as in the cases of the actual disease. Dilutions of 1:80 gave good reactions while in higher dilutions only exceptionally was the clumping clear cut.

The complement fixation tests were not as constantly positive in the vaccinated cases as the agglutinin reaction; the former occurred only in sixty per cent of the cases. It was evident, however, that at least a certain amount of antisubstances or sensibilizators occurred, which justified the employment of vaccin as a prophylactic. It was considered that even though the protective factors stimulated were of low titer and transient in duration, they were of value in the defense against the fulminating epidemic disease.

DISCUSSION AND SUMMARY

During the height of the epidemic of influenza in New Orleans more than five thousand persons were vaccinated by us with a specially prepared protein suspension of the Pfeiffer bacillus. Of this number approximately 90 per cent did not contract influenza, either during the period of the epidemic or its recrudescence which occurred two months afterwards. This percentage compared with that of our group controls (those refusing vaccin) and the city statistics indicate that a considerable degree of protection was established in all those who were completely vaccinated and it is reasonable to suppose that the protection was the result of the inoculation with *B. influenzae* antigen.

The culture used in the preparation of the vaccin was grown upon the surface of hemoglobin nutrient agar at a temperature of 37°C. for thirty-six to forty-eight hours, when the growth was washed off and suspended in normal saline solution and immediately devitalized by being saturated with chemically pure chloroform. In our experience the highest potency vaccin was obtained from the influenza culture, which was killed with chloroform. With this agent the viability of the bacilli was destroyed in a few seconds without apparently causing any deleterious effect upon the protein immunizing substance. While it is

recognized that chloroform in the presence of water and under the influence of light (actinic rays) in the course of time decomposes and give rise to $COCl_2$ (carbonyl chloride) and HCl (hydrochloric acid), which are poisonous substances, it can be stated that these compounds are not evolved in the chloroform vaccin prepared in the manner herein described. In this connection it may be said that the devitalizing agent is left in contact with the saline suspension of bacilli for a short period of time and is then completely removed from the mixture.

In regard to the behavior of chloroform upon the vaccin we believe that it devitalizes the bacilli by rapidly absorbing the water and in consequence increasing the permeability which results first in plasmolysis then rupture of the bacterial cell, liberating without effecting in any manner its toxic moiety. There is nothing to show that chloroform *per se* has any direct chemical action upon bacterial protein whether alone or in combination with water and sodium chloride. On the other hand, this cannot be said for heat and the phenol derivatives when used as vaccine devitalizing agents. Here the destruction of bacterial life is due to a direct action upon the protoplasm, which is coagulated to a greater or lesser extent and in consequence there is destroyed at least a part of the antigenic property of a protein (7, 8). Therefore, in our opinion chloroform as a germicide in the preparation of vaccin has distinct advantages over all other agents commonly employed. The use of heat, tricresol, etc., for killing the culture, while effective from a germicidal standpoint, injures to some degree the immunizing property of the protein and particularly is this the case with *B. influenzae*.

Influenza vaccin, freshly prepared with strict attention to preserving its maximum amount of antigenic value, will excite in the human host the production of specific immune bodies, the degree depending not so much upon the quantity as the quality of the antigen injected. Therefore, the freshness of the vaccin and particularly the method of its preparation are factors of paramount importance if results are to be obtained in the prophylaxis against epidemic influenza. We believe that failure to obtain sufficient protection is, to a large extent, the fault of the

vaccin employed. A contrast study of the results obtained with influenzal vaccins devitalized by heat, tricresol and chloroform will show a wide variation in antigenic effectiveness. The culture killed by heat at 56°C. is practically worthless as an antigen while that prepared by the phenol derivatives even in 0.25 per cent strength is altered in its power to produce antibodies.

While our total number of vaccinated persons is too few to permit of the statement that *B. influenzae* vaccin is a constant specific in the prevention of the disease clinically known as epidemic influenza, it can be said, however, that the results are interesting and significant from the standpoint of prophylaxis.

While the duration of specific antibodies in the blood of the host varies within wide limits for different individuals, it may be said that these substances remain in the circulation for a period of ten weeks on the average, as shown by the agglutination and complement fixation tests. In many isolated cases, however, these tests are positive six months after vaccination.

There occurred in all of the individuals vaccinated a local reaction at the site of inoculation. Usually this was simply a small sharply circumscribed area of erythema; however, in fully 30 per cent of individuals the reaction involved a greater area and there was considerable oedema of the subcutaneous tissues in the vicinity of the inoculation site. Even in the more severe type of local reaction there was a complete subsidence in three to five days.

Constitutional effects following the administration of the vaccin were noted in 90 per cent of the individuals receiving it. In 30 per cent of the cases this host response simulated in symptom-complex the early toxemia of true influenzal infection; however, the reaction subsided in six to twelve hours after the onset. This type of reaction was so striking in its analogy to the early stage of the natural infection that it strongly suggested proof of the relationship between the clinical diseases known as influenza and the artificial toxemia produced by the injection of the Pfeiffer bacillus vaccin.

From the observations herein reported for our series of vaccinations we believe there is every indication that the influenza

protein employed gives rise to the production of protective substances and therefore justifies its use in the prophylaxis of epidemic influenza.

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THE POISONS OF THE INFLUENZA BACILLUS

JULIA T. PARKER

From the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York

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It was shown in a preliminary report (1) that the filtered blood broth cultures of *B. influenzae* are toxic for rabbits. It was stated that the serums of rabbits which had been immunized to the poison possess neutralizing power for the poison. Whether this poison belonged to any known class of poisons was not established. It is the purpose of this paper to give in greater detail the work done to date on this poison of the influenza bacillus with especial regard to its possible classification.

EXPERIMENTAL

Preparation of media

The poison is produced by the growth of *B. influenzae* on several different mediums, in fact, on any medium which contained rabbit hemoglobin in sufficient quantities. The medium, however, from which the poison for this work was obtained, was made as follows:

Veal infusion broth was prepared in the usual way with the exception that an extra amount (2 per cent) of peptone was used; it was found that the Parke, Davis and Company peptone gave the best results; the required amounts of broth were measured into Erlenmeyer flasks and sterilized by the fractional method; the final hydrogen ion concentration should have a pH of from 8 to 8.2; to these flasks enough steril defibrinated rabbit's blood was added to make it 5 per cent of the whole; the flasks were then heated in a water bath at 75°C. until the blood coagulated on

standing; this requires from three to five minutes after this temperature (75°C.) had been reached.

This medium is called for convenience the dark medium. The dark medium and the greater part of the others enumerated below were prepared with the idea of getting rid of the bactericidal action of the rabbit serum either by discarding the serum or by heating it. Most of the following mediums were not used in this work, not because very toxic products could not be produced from them, but because of the much larger amounts of hemoglobin they contained after filtration. Although the amount of hemoglobin contained in the original inoculation was not toxic per se, it was possible that it might be where larger doses were given in immunizing the rabbits and where multiple lethal doses of the poison were tried out. Another reason for using the dark medium was that when filtered it could even be boiled without producing a coagulum, a necessary factor in some of the experiments performed below.

Other methods used

1. Washed rabbit red cells (hemolyzed with distilled water 1–3), 1 to 5 parts of broth.
2. Same as 1 except that the red cells were not washed.
3. Washed red cells hemolyzed with ascitic fluid and broth.
4. Rabbit whole defibrinated blood (not hemolyzed) with broth 1–5.
5. Rabbit red cells (not hemolyzed) with broth.

Very slight if any poison could be produced from the three following mediums—which we believe is explained by the fact that the organisms grew very poorly, or not at all.

6. Same as 1 except that the broth contained 1 per cent glucose.
7. Same as 2 except that the rabbit cells were replaced by sheep cells.
8. Broth and ascitic fluid (no hemoglobin).

It may be noted that the rabbits which were immunized with poisons produced on the mediums 1, 2 and 3 were resistant to the poisons produced on the dark medium.

Strains used

The cultures used were of very small Gram negative pleomorphic bacilli which only grew in the presence of hemoglobin.

Although we obtained poisons from seven different strains of *B. influenzae,* most of this work was carried out with only two strains, 9 and E. We employed these two strains especially because we had found that they produced regularly the most toxic filtrates. It was found, moreover, that an immunity against a poison produced from any one strain will protect against a poison from any other strain. It was also found that an immune serum produced by inoculating rabbits with the poison from any two strains will neutralize *in vitro* at least one lethal dose of the poison from any other strain. An illustration of this latter point is given in table 1.

TABLE 1

Showing the protective action of an antiserum produced by inoculation with two strains against any other strain of B. influenzae

RABBIT	WEIGHT	POISONS	SERUM	REMARKS
	grams			
1	1600	5 cc. 1	1 cc. 340	Lived
2	1580	5 cc. 1	1 cc. 340	Lived
3	1600	5 cc. 1		Dead 1 hour 50 minutes
4	1580	5 cc. 2	1 cc. 340	Lived
5	1595	5 cc. 2	1 cc. 340	Lived
6	1610	5 cc. 2		Dead 1 hour 50 minutes

Poisons 1 and 2 were poisons produced from two strains which Dr. Zinsser brought back from France. Serum 340 was from a rabbit which had nine injections of 9 and E, its last three injections being of 15 cc. each. The animal had been bled six weeks previous to this experiment. The mixtures were incubated at 37°C. for one-half hour before injection.

INOCULATION OF MEDIA

Incubation and filtration after inoculation

Erlenmeyer flasks containing relatively small amounts of the dark medium, which had previously been tested for sterility, were inoculated with one slant of *B. influenzae* for every 50 cc. of the medium. The flasks were incubated at 37°C. for from six to twenty-four hours. It is advisable to shake the flasks several times during this time. After incubation the final pH should be between 7.2 and 7.6. If the poison has a greater acidity than this, it will only have a slight toxicity.

After incubation the cultures were centrifugalized and the supernatant fluid filtered through a Berkefeld candle. The filters used were found impervious to the influenza bacilli and remained so during this work.

Symptoms

The incubation period in rabbits inoculated intravenously with a lethal dose of the poison is invariably thirty to forty minutes. At this time the breathing rate increases, followed usually by very severe dyspnoea. There may or may not be great weakness. An hour after the inoculation the animal is extremely sick. The temperature has risen a little at this time but not higher than 104°F. There are usually muscular twitchings in the neck. The animal may be flattened out on his abdomen either from weakness or from pain. This pain may or may not be followed by diarrhoea. The animal gets progressively worse; he is weaker; the temperature falls below normal and he usually dies within three hours after the injection with or without convulsions.

If a sublethal dose is given, the incubation period is longer, the symptoms are milder and the temperature usually rises to 106° to 107°F. in one to three hours. There is almost invariably a great loss of weight in these animals, this loss being proportional to the dose and sickness of the animal.

If the injections are given intraperitoneally the rabbit takes two to three hours to sicken and the symptoms are very light

even when a larger dose of the poison is given. Subcutaneously injected, the poison is not toxic. At the point of inoculation there may be a sloughing off of the skin one to two weeks later.

The poison is not toxic for guinea-pigs when injected intravenously in 0.5 cc. doses and it does not kill white mice when 2 cc. are given either subcutaneously or intraperitoneally.

PROPERTIES OF THE POISON

The original toxicity of the poison may be preserved for two days if kept frozen solid, otherwise it deteriorates very quickly, even after two or three hours standing. For this reason the toxicity of the poison must be tested and the experiments made on the same day. If 2 cc. of a poison killed a 1400-gram rabbit within three hours we considered it a good one. In most of the experiments given below, the poison was tested in this way before it was used. To safeguard against the possible variation in the resistance of rabbits and against possible deterioration of the poison, two lethal doses were usually given.

A few experiments were done on the effect of heat on the poison. They were not convincing. It is possible that there are two poisons concerned, one thermolabil, destroyed when heated to 75°C. for one-half hour, the other thermostabil, not destroyed even when it is boiled. This thermostabil toxicity may be due to extracted bacterial substances which all these filtrates must contain. More will be said of this later.

The experiments in table 2 were directed to this point.

The poison is precipitated with six volumes of absolute alcohol and appears to retain its original toxicity.

This was demonstrated in the following way: six volumes of absolute alcohol were added to 6 cc. of a poison whose lethal dose was 5 cc. The resulting brownish precipitate was centrifugalized off and dried. It was then taken up with 6 cc. of isotonic salt solution in which it completely dissolved.

A rabbit, weighing 1030 grams, was inoculated with this solution and died with typical symptoms in one hour and twenty minutes. The experiment was repeated with another poison with the same result.

The dark medium is not toxic in itself. This is shown in the following experiment:

The medium was prepared as usual and incubated for twenty-four hours to insure sterility. It was then separated into two flasks A and B. Flask A was inoculated; flask B was not inocu-

TABLE 2

Showing the effect of heat on the poison of B. influenzae

RABBIT	WEIGHT	POISON	REMARKS
		Experiment 1	
	grams		
7	1450	5 cc. A, unheated	Very sick 35 minutes after inoculation. Dead 2 hours, 10 minutes
8	1350	5 cc. A, heated at 48°C. ½ hour	Sick 1 hour after inoculation. Recovered
9	1310	5 cc. A, heated at 55°C. ½ hour	Slightly sick 1 hour after inoculation. Recovered
		Experiment 2	
10	1620	3 cc. B, unheated	Very sick 40 minutes after inoculation. Dead in 5½ hours
11	1900	5 cc. B, heated at 56°C. ½ hour	Very sick 40 minutes after inoculation. Recovered
12	1930	10 cc. B, heated at 56°C. ½ hour	Very sick 30 minutes after inoculation. Recovered
13	1860	5 cc. B, heated at 70°C. ½ hour	Slightly sick 1 hour 15 minutes after inoculation. Slightly sick all the afternoon
14	1950	10 cc. B, heated at 70°C. ½ hour	Slightly sick 1 hour 15 minutes after inoculation. Slightly sick all the afternoon
15	1850	10 cc. B, boiled 5 minutes	Slight labored breathing 1 hour 30 minutes. Slightly sick all the afternoon

lated. Both flasks were incubated for eighteen hours at 37°C., and then centrifugalized and filtered. The toxicity of the two filtrates was tested with rabbits. The results of these tests are presented in table 3.

This protocol shows clearly that the filtrate of the medium is not toxic in itself.

TABLE 3

Showing that the toxicity of the filtrate from cultures of B. influenzae is not a property of the culture medium

RABBIT	WEIGHT	MATERIAL INJECTED	WEIGHT AFTER 24 HOURS	DIFFERENCE IN WEIGHT	RESULTS
	grams		*grams*		
16	1250	2 cc. A			Dead in 2 hours
17	1250	2 cc. B	1310	60 grams gain	Not sick
18	1475	10 cc. B	1520	45 grams gain	Not sick
19	1775	15 cc. B	1755	20 grams loss	Not sick

EXPERIMENTS WITH ANTISERUM TO THE POISON

The serum was produced by injecting rabbits with increasing doses of the poison. The serum of rabbits that had withstood at least four to five lethal doses (15 to 20 cc.) of the poison was used in the following tests. The injections were given five to eight days apart and the amounts were usually increased as follows:

	cc.		*cc.*
First injection	2	Fifth injection	12
Second injection	5	Sixth injection	15
Third injection	8	Seventh injection	18
Fourth injection	10	Eighth injection	20

The rabbits were bled five to ten days after the last injection. A horse also was immunized.

The experiments were carried out by three different methods.

A. By mixing one to two lethal doses of the poison *in vitro* with the serum before injection.

B. By giving the serum fifteen minutes before or fifteen minutes after the injection of the poison.

C. By mixing multiple lethal doses of the poison with serum before injection.

A considerable number of tests were made with the use of the first procedure; namely, that of mixing one to two lethal doses of the poison with serum and incubating the mixtures at 37°C. for one-half hour before inoculation. Five of these protocols are given in table 4.

In this experiment 1 cc. and 2 cc. of immun serum 308 detoxicated a poison that killed the four control rabbits in two hours or less. Normal serum is seen to have no effect on the poison.

Three out of four different immun serums neutralized a poison that killed the two controls in eight to twenty hours. The protected rabbit that died (rabbit 27) survived the controls seven days.

The experiment shows that immun serum 531 protected in 1.5 cc., 1 cc. and 0.5 cc. amounts, but not in 0.25 cc. The controls died in one and a half hours and five and a half hours.

Experiment 4 is similar to experiment 1, table 4, except that the poison was not as toxic and that horse instead of rabbit immun serum was used. It is seen that normal horse serum also has no detoxicating effect on the poison.

Here the horse immun serum neutralized the poison which killed the two controls in three hours and forty-five minutes, and three hours and thirty-five minutes in 0.25 cc. amounts.

In each case the control animals getting the poison alone and poison and normal serum were injected last in order to give the tubes containing these mixtures the benefit of a few minutes extra standing.

In the third and fourth experiment in table 4 the loss of weight in the animals that survived was a good indication of their sickness. It is seen that the animals that received the greatest amount of immun serum lost the least weight and *vice versa*. This is almost always the rule when the rabbits are of approximately the same weight when injected. The weights in experiment 5, table 4, are a rare contradiction to this rule.

Experiment 4, table 4, is given as it shows that the horse serum had some protective action even though the horse at this time had only had two injections of the poison. The horse had had 13 injections when the serums used in experiment 5, table 4, was obtained.

It is seen from the foregoing protocols that the immun serum from both rabbits and from a horse neutralized *in vitro* at least one lethal dose of the poison. It is also seen that normal serum has no such effect.

TABLE 4A

Showing the neutralizing power of antisera prepared against the poison of B. influenzae

Experiment 1

RABBIT	WEIGHT	POISON	SERUM	RESULTS
	grams			
20	1450	5 cc. A	2 cc. 308	Lived
21	1530	5 cc. A	1 cc. 308	Lived
22	1500	5 cc. A	2 cc. Normal	Dead 1 hour 15 minutes
23	1530	5 cc. A	1 cc. Normal	Dead 1 hour 45 minutes
24	1500	5 cc. A		Dead 1 hour 20 minutes
25	1550	5 cc. A		Dead 2 hours

TABLE 4B

Experiment 2

RABBIT	WEIGHT	POISON	SERUM	RESULT
	grams			
26	1650	4.5 cc. B	1 cc. 308	Lived
27	1650	4.5 cc. B	1 cc. 368	Dead 8 days
28	1660	4.5 cc. B	1 cc. 369	Lived
29	1670	4.5 cc. B	1 cc. 324	Lived
30	1680	4.5 cc. B	1 cc. Normal	Dead 8 hours
31	1710	4.5 cc. B		Dead 20 hours

TABLE 4C

Experiment 3

RABBIT	WEIGHT	POISON	SERUM	RESULTS	WEIGHT AFTER 24 HOURS	LOSS
	grams				*grams*	*grams*
32	1540	5 cc. C	0.25 cc. 531	Dead 1 hour		
33	1540	5 cc. C	0.5 cc. 531	Lived	1175	365
34	1520	5 cc. C	1.0 cc. 531	Lived	1360	160
35	1520	5 cc. C	1.5 cc. 531	Lived	1500	20
36	1580	5 cc. C		Dead $5\frac{1}{2}$ hours		
37	1600	5 cc. C		Dead $1\frac{1}{2}$ hours		

TABLE 4D

Experiment 4

RABBIT	WEIGHT	POISON	SERUM	RESULTS	WEIGHT AFTER 72 HOURS	LOSS
	grams				*grams*	*grams*
38	1560	5 cc. D	2 cc. Immun horse (3/17)	Lived	1540	20
39	1680	5 cc. D	1 cc. Immun horse	Lived	1330	350
40	1680	5 cc. D	2 cc. Normal horse	Dead during night		
41	1725	5 cc. D	1 cc. Normal horse	Dead during night		
42	1700	5 cc. D		Dead 1 hour 40 minutes		
43	1810	5 cc. D		Dead 7 days	1400	410

TABLE 4E

Experiment 5

RABBIT	WEIGHT	POISON	SERUM	RESULTS	WEIGHT AFTER 24 HOURS	LOSS
	grams				*grams*	*grams*
44	2340	5 cc. E	0.25 cc. Immun horse (5/3)	Lived	2280	60
45	2050	5 cc. E	0.25 cc. Immun horse	Lived	1960	90
46	2100	5 cc. E	0.5 cc. Immun horse	Lived	1920	180
47	2245	5 cc. E	0.5 cc. Immun horse	Lived	2135	110
48	2255	5 cc. E	1.0 cc. Immun horse	Lived	1745	210
49	1830	5 cc. E	1.0 cc. Immun horse	Lived	1640	190
50	2355	5 cc. E		Dead 3 hours 45 minutes		
51	2510	5 cc. E		Dead 3 hours 35 minutes		

Although comparatively little work has been carried out with the serum used prophylactically or therapeutically, still there seems to be little doubt that it is effective here also. Five of the protocols are given in tables 5a, 5b, 5c, 5d and 5e.

In this experiment one of the two rabbits was saved by being

TABLE 5A

Showing the prophylactic and therapeutic action of the antiserum upon the injection of the poison of B. influenzae

Experiment 1

RABBIT	WEIGHT	POISON	SERUM	RESULTS
	grams			
52	1360	5 cc. A	5 cc. 307	Dead 1 hour 20 minutes
53	1380	5 cc. A	10 cc. 307	Lived
54	1400	5 cc. A	5 cc. 308	Lived
55	1410	5 cc. A	10 cc. 308	Lived
56	1400	5 cc. A	5 cc. Normal	Dead 1 hour 20 minutes
57	1410	5 cc. A	10 cc. Normal	Dead 23 hours
58	1400	5 cc. A		Dead 1 hour 55 minutes
59	1440	5 cc. A		Dead 2 hours 7 minutes

TABLE 5B

Experiment 2

RABBIT	WEIGHT	POISON	SERUM	RESULTS
	grams			
60	1475	5 cc. B	10 cc. 410	Lived
61	1450	5 cc. B	10 cc. 410	Dead 3 hours 45 minutes
62	1515	5 cc. B	10 cc. Normal	Dead 1 hour 35 minutes
63	1475	5 cc. B		Dead 1 hour 25 minutes
64	1555	5 cc. B		Dead 2 hours

TABLE 5C

Experiment 3

RABBIT	WEIGHT	POISON	SERUM	RESULTS
	grams			
65	1490	5 cc. C	10 cc. 311	Dead 3 days
66	1520	5 cc. C	10 cc. Mixed immune serum	Dead 24 hours
67	1510	5 cc. C	10 cc. Normal	Dead 1 hour 15 minutes
68	1560	5 cc. C	10 cc. Normal	Dead 1 hour 25 minutes
69	1590	5 cc. C		Dead 41 minutes

TABLE 5D

Experiment 4

RABBIT	WEIGHT	POISON	SERUM	RESULTS
	grams			
70	1500	4 cc. D	10 cc. 345	Lived
71	1520	4 cc. D	10 cc. 345	Lived
72	1530	4 cc. D	10 cc. Normal	Dead 14 days
73	1546	4 cc. D	10 cc. Normal	Dead 1 hour 20 minutes
74	1535	4 cc. D		Dead 45 minutes
75	1545	4 cc. D		Dead 1 hour 25 minutes

TABLE 5E

Experiment 5

RABBIT	WEIGHT	POISON	SERUM	RESULTS
	grams			
76	1540	5 cc. F	9 cc. 410	Lived
77	1600	5 cc. F	9 cc. 343	Lived
78	1600	5 cc. F	9 cc. Normal	Dead 6 days
79	1680	5 cc. F	9 cc. Normal	Dead 1 hour 5 minutes
80	1620	5 cc. F		Dead 7 days
81	1700	5 cc. F		Dead 6 days

injected with 5 cc. of immun serum fifteen minutes before the injection of the poison. Both rabbits getting 10 cc. of immun serum fifteen minutes after the injection of the poison survived. The four controls died; one of these rabbits (no. 57), which received 10 cc. of normal serum fifteen minutes after the inoculation of the poison, lived twenty-one hours longer than the other three controls. This result shows that in this case the normal serum may have conferred a slight amount of protection.

The poison in this experiment was a very toxic one. One out of two of the treated animals lived; the other died in three hours and forty-five minutes, surviving the three controls by about one hour. The normal serum did not have any effect on the poison.

This is also a very violent poison. All of the five rabbits succumbed; however, the two treated animals lived a relatively much longer time than the controls. The normal serum is seen to confer no protection.

In this experiment the two treated animals survived. Three of the four controls died in less than one hour and a half. The other one (no. 72), which got the normal serum, lived for fourteen days. Here again the normal serum seems to have protected the rabbit to a certain degree.

In this experiment 9 cc. of the two different immun serums saved the two rabbits, while the four controls died. In this case normal serum did not show any nonspecific protective action.

In this series all of the rabbits except numbers 52, 54, 56, were inoculated with the specified serum fifteen minutes after the injection of the poison. The latter three rabbits were given the serum fifteen minutes before the injection of the poison.

Table 5 shows that where relatively large amounts of antiserum are give either fifteen minutes before or fifteen minutes after, at least one lethal dose of the poison, the animal is usually saved. Although normal serum exerts occasionally a slight curative power, this effect, can, in no way, be compared with that of the immun serum.

EXPERIMENTS WITH MULTIPLE LETHAL DOSES OF THE POISON

Several attempts were made to neutralize multiple lethal doses of the poison with immun serum, but with little success. Several protocols are give below where relatively large amounts of immun serum have been mixed with multiple lethal doses of the poison. It will be seen that less than 50 per cent of the animals that received such mixtures survived.

Five cubic centimeters of the poison in this experiment was probably more than one minimal lethal dose. Although both rabbits died, the rabbit getting the 15 cc. of the poison and the immun serum survived the control by twenty hours, showing some neutralization.

In this experiment one of the two rabbits getting 3 minimal lethal doses of the poison and 6 cc. of the immun serum lived, while the two controls died.

This experiment is similar to the preceding one. However, the immun serum showed protection even in rabbit 88 as this

TABLE 6A

Showing the failure of the antiserum to neutralize multiple lethal doses of the poison of B. influenzae

Experiment 1

RABBIT	WEIGHT	POISON	SERUM	RESULTS
	grams			
82	1450	15 cc. A	6 cc. 531	Dead 22 hours
83	1400	5 cc. A		Dead 1 hour 30 minutes

TABLE 6B

Experiment 2

RABBIT	WEIGHT	POISON	SERUM	RESULTS
	grams			
84	1340	15 cc. B	6 cc. 523	Dead during night
85	1400	15 cc. B	6 cc. 523	Lived
86	1440	5 cc. B		Dead 3 hours
87	1410	5 cc. B		Dead during night

TABLE 6C

Experiment 3

RABBIT	WEIGHT	POISON	SERUM	RESULTS
	grams			
88	1620	12 cc. C	6 cc. 523	Dead 7 days
89	1600	12 cc. C	8 cc. 523	Lived
90	1680	4 cc. C		Died during night
91	1620	4 cc. C		Dead 1 hour 25 minutes

animal survived the controls by at least six days. The mixtures were incubated for 30 minutes at 37°C. before injection.

It is well known that animals of the same species may differ in their ability of producing antibodies against the same antigen. This was also the case with our rabbit immun serums which we tested in this respect in several instances. An extreme example of this point is given below.

The mixtures were incubated as usual before inoculation. It will be seen that serum 536, which was obtained from a rabbit that had had seven injections of poison (the last two being 18 cc. and

20 cc. respectively), had no neutralizing action, while serum 510, obtained from a rabbit which had seven injections (the last two being 15 cc. each) was relatively active in this respect. This led to a test of the agglutinins, precipitins, and complement fixing antibodies of these two serums to discover whether there was any relation between these antibodies and the neutralizing activities of a serum.

Serum 536 was found to agglutinate completely *B. influenzae* 9 and E +++ in a dilution of 1–40; serum 510 was +++ in a dilution of 1–100. No precipitins could be demonstrated in

TABLE 7

Showing a difference between two rabbits in the production of the power of neutralizing the poison of B. influenzae

RABBIT	WEIGHT	POISON	SERUM	RESULTS
	grams	*cc.*		
92	1200	5.0	1.0 cc. 536	Dead during night
93	1200	5.0	0.5 cc. 536	Dead during night
94	1320	5.0	0.25 cc. 536	Dead during night
95	1200	5.0	1.0 cc. 510	Not sick. Lived
96	1280	5.0	0.5 cc. 510	Not sick. Lived
97	1280	5.0	0.25 cc. 510	Sick. Lived
98	1350	5.0		Dead during night
99	1320	5.0		Very sick. Lived

either serum. Serum 536 (no. 10 in table 8) fixed complement completely, ++++, in 0.01 cc. amounts, while serum 510 (no. 9 in table 8) fixed completely in 0.001 cc. amounts. In these two instances, therefore, the complement fixing antibodies ran parallel with the power of neutralization. This was also the case with no. 4, table 8 (compare with neutralizing properties, table 4, experiment 5) and with no. 12, table 8, which we found neutralized easily in 0.25 cc. doses, at least one lethal dose of the poison.

A number of recent and old immun serums were then tested for their complement fixing power. Some of the results obtained are given in table 8.

The antigen was made of cultures 9 and E and one-third of the anticomplementary dose was used in the tests in table 8. The animals were bled on the dates noted in table 8.

It is seen from this table that, with few exceptions, the oldest serums have the least power of fixing complement. In only one case did we try the neutralizing action of an old serum. Although this serum had previously neutralized a lethal dose of the poison

TABLE 8

Showing the complement-fixing power of various antisera produced against the poison of B. influenzae

		AMOUNT OF SERUM							
		0.1 cc.	0.01 cc.	0.001 cc.	.0075 cc.	.0005 cc.	.00025 cc.	0.00001 cc.	REMARKS
Horse									
3/17	1	++++	+++	0					
3/29	2	++++	++++	++++	+++	0	0	0	0.1 and 0.01 slightly anti-complementary
4/19	3	++++	++++	++++	++++	++	0	0	0.1 and 0.01 slightly anti-complementary
5/3	4	++++	++++	++++	++++	++++	0	0	0.1 and 0.01 slightly anti-complementary
Rabbit									
536, 5/3	5	++++	±	0	0				
308, 12/6	6	±	0	0	0				
306, 12/21	7	±	0	0					
340, 2/11	8	++++	++++	+					0.1 slightly anticomplementary
510, 5/27	9	++++	++++	++++	+++	+	0	0	
536, 3/27	10	++++	++++	±					
533, 4/24	11	++++	++++	±	0	0	0	0	
572, 6/11	12	++++	++++	++++	++++	++++	++++	±	

in 1 cc. amounts, when tested again four months later, it had lost all this power. These facts are noted here because they seem to indicate the possibility that the neutralizing power of a serum runs parallel with its complement fixing power. More light is thrown on this phenomenon by experiments cited below.

Although it seemed very improbable that any other antiserum produced by inoculating some other organism into rabbits could protect against the influenza poison, still in order to set aside any possible nonspecific action of an immun serum produced from another organism, 1 and 2 cc. of a high titer typhoid rabbit serum were incubated with a lethal dose of the poison of *B. influenzae*. There was no neutralization.

THE POISONS IN THE VACCIN OF B. INFLUENZAE

The question as to whether our poison could be produced from dead bacteria, fresh or autolyzed, whole or filtered was next investigated. It seemed possible that the poison of *B. influenzae* might be related closely to those produced from dysentery (Shiga Kruse), typhoid and cholera cultures by Kraus and Doerr (2), Kraus and Stenitzer (3), and Kraus (4), respectively. These observers found that their poisons were contained in bacterial extracts as well as in broth filtrates and that antiserum produced by immunizing either with the extracts or with the filtrates produced equally good serums against the poison. Pfeiffer (5) in his original work on *B. influenzae* found that the same symptoms were caused in rabbits by the inoculation of dead or living cultures and that the same amount of culture was necessary to kill in either case. It was, therefore, thought advisable to test out the toxicity of vaccins of *B. influenzae*.

EXPERIMENTS WITH VACCIN OF B. INFLUENZAE

The vaccin was prepared as follows. The heavy eighteen hour growth of *B. influenzae* (9 and E) on coagulated rabbit blood slants was washed off with isotonic salt solution, 2 cc. to each tube. 0.1 cc. of normal NaOH was added to each 100 cc. of vaccin. After heating for one-half hour at 58°C. the bottle was shaken in the shaking machine for one and one-half hours. Microscopic examination of such material at the end of this time showed the bacilli to be well broken up and staining poorly. The autolyzed vaccin (B) was prepared in the same way, but was incubated at 37°C. for twenty hours in addition.

Both vaccins were very opaque and sedimented extremely slowly.

EXPERIMENTS WITH FRESH VACCINS

It will be noticed in table 9 that antigen A (fresh vaccin) is very little toxic. The difference in resistance of rabbits to the bacterial antigen is well illustrated in this experiment by the fact that rabbit 102, which had received an injection of only 0.5 cc., was much sicker and lost a correspondingly greater amount of weight than did the other rabbits. This variability in rabbits was shown in almost all our experiments with the vaccin and was in marked contrast to the experiments with the broth poison, where the resistance was fairly even.

TABLE 9

Determination of the toxicity of a vaccin of B. influenzae

RABBIT	WEIGHT	VACCIN	RESULT	WEIGHT AFTER 24 HOURS	LOSS
	grams			*grams*	*grams*
100	1430	2.0 cc. A	Sick 1 hour after injection; quick breathing. Better after 2 hours	1250	180
101	1400	1.0 cc. A	Slightly sick 1 hour after injection	1235	165
102	1380	0.5 cc. A	Very sick 1 hour after injection lying on abdomen. Diarrhoea. Sick all the afternoon	1105	275
103	1380	0.25 cc. A	Very little sick	1235	155

EXPERIMENT WITH AUTOLYZED VACCIN

The vaccin B (autolyzed vaccin) was possibly slightly more toxic than vaccin A. The symptoms of the rabbits were the same in either case. It was our intention to learn the lethal dose of this antigen and see whether a good immun serum to the broth poison would protect against it. This was tried twice without showing any neutralizing activity of the serum. One protocol is given below, table 10. One cubic centimeter of the horse serum which protected in 0.25 cc. amounts (see table 3, experiment 5) was used in the test.

The mixtures were incubated at 37°C. for one hour.

EXPERIMENTS WITH VACCIN FILTRATE

The Berkefeld filtrate appeared to have about the same toxicity as the whole vaccin.

A dose of 3.6 of vaccin A filtrate was given to a rabbit weighing 1150 grams. He was sick all day and died during the night. A smaller dose (2 cc.) was given to a 900-gram rabbit. This animal survived.

The toxic properties of the vaccin are therefore filtrable, a fact which, of course, was to be expected, but which we thought best to prove by experiment before going on.

TABLE 10

Showing the failure of a broth poison antiserum to protect against the toxic action of a vaccin of B. influenzae

RABBIT	WEIGHT	ANTIGEN	SERUM	RESULTS
	grams			
104	1660	3 cc. B	1 cc. Immune horse	Very sick but recovered
105	1800	3 cc. B	1 cc. Immune horse	Very sick. Dead during night
106	1870	3 cc. B		Very sick. Diarrhoea. Dead 3 days
107	1810	3 cc. B		Sick, but recovered

EXPERIMENT WITH BOILED FILTRATES OF THE ANTIGEN

As the broth poison was still toxic even when boiled it was thought advisable to test the vaccin filtrate under the same conditions. In this experiment the vaccin was prepared as before with the exception that the bacilli were killed by shaking with toluol instead of by heat. It was found that the boiled vaccin filtrate was slightly more toxic than the unboiled. An example of this is given in table 11.

Five cubic centimeters of the filtrate corresponded to one slant of culture.

From the foregoing experiments with bacterial vaccin, it does not seem likely that the bacterial extractive substances as obtained above possess more than slight toxic properties and these would appear in the broth filtrates together with the true toxins.

It seemed possible that the toxicity remaining after heating to 75°C. or over might be due to these substances.

With the hope of throwing more light upon this possibility, the following experiments were carried out (tables 12 and 13).

The broth poison was heated to 75°C. for one-half hour before the serum was added. The mixtures and poisons alone were incubated as usual at 37°C. for one-half hour before injection.

An explanation is required before discussing this experiment, table 12. On reviewing this work, there appear to be two possibilities in regard to the poison of *B. influenzae*.

TABLE 11

Showing the effect of boiling on the toxicity of the filtrate from the vaccin of B. influenzae

RABBIT	WEIGHT	VACCINE FILTRATE	RESULTS	WEIGHT AFTER 24 HOURS	LOSS
	grams			*grams*	*grams*
108	1200	5 cc.	Very sick. Recovered	1180	20
109	1190	5 cc. Boiled	Very sick. Dead 4 days	1120	70

1). That the filtrate contains only one poison; that heating it merely destroys part of its toxicity and that the remaining toxicity is the same as its original toxicity except that it is attenuated.

2). That this poison of *B. influenzae* contains two poisons: one, a true toxin, which is only produced during the growth of the bacilli on blood broth, which is filtrable, and thermolabil, and which may be neutralized in multiple lethal doses by the immun serum; the other, also filtrable, but thermostabil and not neutralized by the immun serum.

Considering table 12 from this point of view it is seen, in the first place, that except for a slight amount of sickness and considerable loss of weight (220 grams) 5 cc. of serum 572 was able to neutralize three lethal doses of the unheated broth poison (see rabbit 110). On the other hand, although 15 cc. of the heated broth poison is only one lethal dose (see rabbit 111), rabbit 112, which was inoculated with 15 cc. of the heated poison

(one lethal dose) and 5 cc. of serum 572, was, if anything, sicker than rabbit 110.

If the immun serum had the same effect on the heated poison as on the unheated, one would have expected the heated poison given rabbit 112 to have been entirely neutralized. But rabbit 110 was, if anything, less sick and lost less weight than rabbit 112. Hence, it seemed most likely that the immun serum

TABLE 12

Showing the neutralizing effect of antiserum 572 on the poisonous action of the heated and unheated broth poison of B. influenzae

RABBIT	WEIGHT	POISON	SERUM	WEIGHT AFTER 24 HOURS	LOSS	RESULTS
	grams			*grams*	*grams*	
110	1600	15 cc.	5 cc. 572	1380	220	After 1 hour diarrhoea slightly sick all the afternoon. Lived
111	1610	15 cc. heated		1170	440	After 45 minutes sick, dyspnoea. Sicker than rabbit 108. Dead 6 days
112	1620	15 cc. heated	5 cc. 572	1350	270	After 55 minutes sick. Diarrhoea. As sick as rabbit 110. Dead 6 days
113	1640	5 cc.				After 40 minutes sick. Diarrhoea. Lying on side. Very sick all the afternoon. Dead 21 hours
114	1660	5 cc.				After 40 minutes sick. Dyspnoea. Very sick all the afternoon. Dead during the night.

neutralized a poison that the heated broth did not contain. The fact that rabbit 111 was sicker and lost more weight than rabbit 112 seems to indicate that the serum was in a slight way protective in this case also. It was thought that this might be a nonspecific action aud that normal serum would possess this same power, just as it may have helped in a slight degree when the normal serum was used curatively (see table 5). This was investigated further in table 13.

The following points are brought out in table 13. It is seen that 5 cc. is the lethal dose of the poison and 20 cc. of the heated poison. The only rabbits that survived were nos. 115 and 118, the former getting four lethal doses of the unheated poison and 6 cc. of serum 572 and the latter one lethal dose of the heated poison and the same amount of serum. Rabbit 118 was sicker than rabbit 115, which corroborates the findings in table 12; namely that the immun serum has not the same effect on the unheated as the heated poison. No other deduction can be made from this experiment. Normal serum had some detoxicating effect on the unheated poison (see rabbit 116) while it had the reverse effect in the case of the heated poison (see rabbit 119) in which case the result was probably due to unusual susceptibility of this rabbit to the heated poison.

TABLE 13

Comparison of the neutralizing effect of normal and immun serum upon the poisonous action of the heated and unheated broth poison of B. influenzae

RABBIT	WEIGHT	POISON	SERUM	RESULTS	WEIGHT AFTER 22 HOURS	LOSS
	grams				*grams*	*grams*
115	1500	20 cc.	6 cc. 572	Lived	1380	120
116	1500	20 cc.	6 cc. Normal	Dead 4 days	1310	190
117	1550	20 cc. heated		Dead 5 days	1450	100
118	1510	20 cc. heated	6 cc. 572	Lived	1350	160
119	1515	20 cc. heated	6 cc. Normal	Dead 1 hour 20 minutes		
120	1560	5 cc.		Dead 1 hour 15 minutes		
121	1580	5 cc.		Dead 1 day	1400	180

EXPERIMENTS WITH ANTISERUM TO THE VACCIN

These experiments were undertaken to discover whether a serum obtained by immunizing rabbits with the vaccin could detoxicate the broth poison *in vitro*. If this could not be done it would be an added point in favor of the theory that the toxicity of the vaccin and broth filtrates were different.

It was difficult to immunize rabbits against the vaccin. Four cubic centimeters of the vaccin (the bacilli from two slants)

was the largest quantity injected. However it was comparatively easy to obtain a high titer serum. The serum used in these tests fixed complement in a quantity of 0.00025 to 0.00001 cc. Our best antiserums to the broth poison fixed in 0.0005 cc. to 0.00025 cc. and detoxicated at least one lethal dose of the broth poison in a quantity of 0.25 cc. Two protocols of this work are given in tables 14 and 15.

TABLE 14

Showing that the antiserum to the vaccin of B. influenzae lacks power to detoxicate the broth poison

RABBIT	WEIGHT	POISON	SERUM	RESULTS	WEIGHT AFTER 24 HOURS	LOSS
	grams	*cc.*			*grams*	*grams*
122	1410	5	1 cc. 567	Very sick. Recovered	1230	180
123	1510	5	0.5 cc. 567	Dead 45 minutes		
124	1380	5		Dead 55 minutes		
125	1420	5		Very sick. Recovered	1325	95

TABLE 15

Showing that the antiserum to the vaccin of B. influenzae lacks power to detoxicate the broth poison

RABBIT	WEIGHT	POISON	SERUM	RESULTS
	grams	*cc.*		
126	1350	5	1 cc. 548	Dead 5 days
127	1280	5	1 cc. Normal	Dead 1 day
128	1300	5		Dead 4 days
129	1229	5		Dead 2 days

Serum 567 obtained from a rabbit that had had three injections of vaccin and whose serum fixed complement completely in a quantity of 0.00025 cc. and partially in a dose of 0.0001 cc. The broth poison was made, as usual, on the dark medium.

In this experiment (table 15) one control and one treated animal died. No protection can be made out here, for of the two animals that survived the one getting the 1 cc. of serum 567 lost more weight than the control.

Serum 548 fixed complement completely in a dose of 0.00025

cc. Although the poison used in the above experiment was a weak one, serum 548 did not save rabbit 126.

From the foregoing experiments it is seen that the power to fix complement bears no relation to the power to neutralize the broth poison. Thus, it appears that in antiserums produced by immunizing with this poison, the power to fix complement and to neutralize the poison run parallel, not because these antibodies are in any way related to each other in the detoxication of the poison, but because rabbits that can produce a serum of high titer for fixing complement are also the best for the production of antitoxin.

The last point to be considered was whether the filtrate of the dark medium after incubation with dead *B. influenzae* would be poisonous. The poisons in this case would be in the nature of proteotoxins.

This was tried out three times and in one case the dead bacterial filtrate made a rabbit sick. This protocol is given below.

Experiment. Two hundred cubic centimeters of the dark medium was prepared as usual and was separated into two flasks A and B, 100 cc. to each flask. A was inoculated with 1 slant each of E and 9 alive; B was inoculated with 1 slant each of E and 9, which had been heated to 58°C. for one-half hour and proved steril.

After eighteen hours incubation A and B were centrifugalized and filtered.

Rabbit 130, weight 1690 grams, was injected with 4 cc. of A. Forty minutes after the injection the animal was very sick with severe dyspnea. Later it became weak and died during night.

Rabbit 131, weight 1760 grams, was injected with 4 cc. of B. One hour and twenty-five minutes after injection the animal exhibited weak, rapid breathing. Recovered.

In the other two experiments there was slight if any sickness in the animals given the B filtrate even when twice the amount given the control was injected. We also tried incubating the flasks with dead bacilli for forty-eight hours without, however, observing any increase in toxicity. It is possible that rabbit 131 was especially susceptible to the bacterial extracts which, of course, are contained in small amount in these filtrates and to

which, as previously shown (table 9), rabbits vary greatly in their resistance.

We hope to clear up this point later.

DISCUSSION

In this work an effort has been made to classify the broth poison of *B. influenzae*. To date this has not been possible. It was of first importance to ascertain whether this poison is a true soluble toxin. Although it possesses some of the attributes of such toxins, it lacks others. The fact that the antiserum will detoxicate, *in vitro*, three minimal lethal doses of the poison in only about 50 per cent of the cases, even when an excess of serum is used, speaks against its being a true toxin.

On the other hand there is a possibility that the poison is a combination of two poisons; the first a true soluble toxin, which is produced during the growth of *B. influenzae* and can be neutralized by the antiserum in multiple lethal doses; the second a thermostabil filtrable poison, which is found also in bacterial extracts and against which the antiserum has no powers. According to this conception, the animal receiving multiple lethal doses of the broth poison and the immun serum would not die from the toxin, but from a lethal dose of the thermostabil poison present in the filtrate.

Being unable to prove that the broth poison is a soluble toxin, we next tried to determine whether it could be similar to the dysentery, typhoid, and cholera poisons. In this we also failed, for these latter poisons could be produced as well from dead bacterial extracts as from broth filtrates, which was not possible with our poison.

Not enough work has been done on the possible proteotoxin formation of the Pfeiffer bacillus on the blood broth mediums. The fact, however, that our poison can be neutralized *in vitro* with relatively small amounts and *in vivo* with larger amounts of antiserum, speaks against its being a proteotoxin. It must also be remembered that rabbits can be immunized with great ease to four or five lethal doses of the poison, which has never been done with anaphylatoxin, produced by whatever method.

CONCLUSIONS

1. *Bacillus influenzae* produces a filtrable poison which is lethal to rabbits when given intravenously.

2. The poison is only partly destroyed when heated to 55°C. for one-half hour. When heated to 75°C. for one-half hour or boiled for five minutes, over two-thirds of its toxicity has been lost.

3. Rabbits can be immunized to at least four or five minimal lethal doses of this poison.

4. One-quarter to 1 cc. of the immun serum can neutralize *in vitro* one to two lethal doses of the poison.

5. Five to 10 cc. of the immun serum, when given intravenously fifteen minutes before or fifteen minutes after the injection of one to two lethal doses of the poison, will usually save the rabbit.

6. Five to 8 cc. of immun serum, when mixed *in vitro* with at least three minimal lethal doses of the poison, will save about 50 per cent of the rabbits. Influenza bacterial extracts, fresh or autolyzed, are poisonous to rabbits in relatively large amounts. The symptoms are the same as with a sublethal dose of the broth filtrate. A dose corresponding to one and one-half heavily grown slants of *B. influenzae* will kill 50 per cent of the rabbits injected.

7. The Berkfeld filtrate of the bacterial extracts is nearly as toxic as the extracts themselves. Boiling this filtrate does not destroy its toxicity.

8. The immun serum has no effect *in vitro* even in large amounts in detoxicating the bacterial extracts.

9. Antiserums produced by immunizing with vaccins of *B. influenzae* do not neutralize *in vitro* a lethal dose of the broth poison.

10. While the evidence is by no means conclusive, it seems probable that the poison of *B. influenzae* contains two poisons; the first, the more important one, a true soluble toxin, filtrable, thermolabile, against which antitoxins can be produced; the second, present also in the vaccin of *B. influenzae*, also filtrable, but

differing from the first poison in its thermostability, and in the fact that it is not detoxicated by the antitoxin.

I am much indebted to Dr. James G. Dwyer and Dr. Stewart Polsom for assistance in the inoculations of the horse and to Miss Lucy Pope for technical help with the cultures. I am also much indebted to Mr. James J. May, who has been of the greatest assistance in all the work connected with these experiments.

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ON THE EXISTENCE OF A MULTIPLICITY OF RACES OF B. INFLUENZAE AS DETERMINED BY AGGLUTINATION AND AGGLUTININ ABSORPTION

EUGENIA VALENTINE AND GEORGIA M. COOPER

From the Bureau of Laboratories, Department of Health, New York City

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In the general investigation of the bacteriology of the recent epidemic of influenza, carried out under the supervision of Dr. Wm. H. Park and Dr. Anna W. Williams,[1] it was early seen that one of the most important questions was the identity or non-identity of the strains of *B. influenzae* from epidemic cases. Although the practically uniform presence of *B. influenzae* in actual cases suggested its etiological importance, the further demonstration was needed that the strains encountered were identical before a conclusion was possible. It would be difficult to conceive of a pandemic spreading from country to country, due to different races of a microörganism.

Preliminary observations indicated that the relationship of the strains isolated could be determined most easily by agglutination and agglutinin absorption. It was found that agglutinating sera, active in dilutions of 1–800 to 1–1000, could be produced without great difficulty by intravenous injection of rabbits, starting with live or killed bacilli. The following schedule will serve as a general outline of the method we have employed.

Increasing amounts, first of heated suspensions, later of live suspensions were injected intravenously into rabbits. The doses were increased from an amount equivalent to the growth on $\frac{1}{5}$ of a slant to that on 1 to 2 slants. The organisms were cultivated on "chocolate" agar[2] in 6 by $\frac{5}{8}$ inch tubes with about a

[1] We are indebted to Dr. Charles Krumwiede for direct technical supervision.

[2] Glycerine veal agar, 10 cc.; citrated horse blood, 0.5 cc.; add blood to the boiling hot agar; mix thoroughly and slant.

$1\frac{1}{2}$ inch length of slant surface. Injections were made on three consecutive days, and then a rest period of four days intervened. After two series of injections, on the fourth day following the last injection of the second series, a trial bleeding was tested. If the titer of the serum was satisfactory, the rabbit was etherized and bled to death. If it fell short, the injections were continued as outlined above and trial bleedings were tested on the fourth day after the last injection of each series until the serum was found sufficiently potent. With the majority of strains, the immunization required about four weeks.

The agglutination antigen was prepared by scraping into 0.8 per cent salt solution the growth of a twenty-four-hour culture on "chocolate" medium. Vigorous shaking by hand was sufficient to give an even suspension of the bacilli. Some strains encountered had a tendency to clump spontaneously, but these clumps were extremely unstable, and on slight shaking, could be easily distinguished from serum agglutination. The suspension made on different days but from the same cultures, showed only a slight to moderate degree of variation in regard to their agglutinin ability. In spite of such variations, the general results, as shown in the tables, have been relatively uniform and repeated tests have been sufficient in number to exclude any possibility of error due to this factor.

The technique for the agglutination tests was as follows: The sera were diluted to 0.1 of the final dilutions given in the tables; 0.1 of serum dilution and 0.9 of the bacterial suspension were pipetted into tubes and thoroughly mixed by shaking. Very thin suspensions, that is, suspensions showing only moderate cloudiness, were found to give the sharpest readings and were, therefore, employed. Uniform density was approximated by reading type through the suspension in a test tube 1 inch in diameter. Two cultures failed to agglutinate on the first trial. However, repetition gave sharp results. Differences so marked were most probably due to error in selecting cultures for suspension (confusion of names and numbers). The tests were incubated in the water bath at 45°C. for two hours. Readings

were made at the end of this time; these readings were checked by a second one made after the tests had been in a cold room over night. These readings differed to a negligible degree; some strains showed better agglutination at the first reading and others at the second. The second readings were on the whole more difficult to make than the first, because of the tendency of the organisms to settle to a much larger degree even in the absence of actual agglutination. The earliest readings are reported in the tables. The symbols +, ±, × and − were adopted to show the grades of agglutination. By +, a complete agglutination is indicated. The clumps varied from large to medium in size with different strains, but the supernatant fluid cleared as the clumps settled. By ±, a less complete reaction is indicated; the clumps were of smaller size and the supernatant fluid, after the settling of the clumps, showed slight opalescence. A × is used to indicate still less marked reactions than the preceding, as slight clumping or a settling with no coherent clumps; in short, any trace of reaction occurring beyond that found in the control tubes.

The first strains selected for study, with one exception, were isolated at post mortem from lung or larynx. If there were an epidemic strain it seems that the strains having the ability to extend from the nasopharynx and invade the lung would most likely be the actual epidemic variety rather than an accidentally present parasitic type. As has been shown, the influenza bacillus is very frequently encountered in the nasopharynx of apparently normal individuals as well as in the throats of persons suffering from diseases due to other agents. The preliminary strains were nine in number, and an agglutinating serum was prepared for each. For comparison, a culture of *B. influenzae* (B. I.) isolated in 1914 from the lung at autopsy in a case of pneumonia and a serum prepared against the culture were included. The results of the direct agglutinations are shown in table 1.

The general absence of cross agglutination, or the slight degree when present, as shown in this table, indicates that even among these ten strains there exist dissimilar varieties.

To exclude the possibility that the cross agglutination obtained

TABLE 1*

Direct agglutinations

STRAINS	AGGLUTINATING SERA: Benson 100	Benson 200	Benson 400	Benson 700	Benson 1000	Lee 100	Lee 200	Lee 400	Lee 700	Lee 1000	Sykes 100	Sykes 200	Sykes 400	Sykes 700	Sykes 1000	Laverdi 100	Laverdi 200	Laverdi 400	Laverdi 700	Laverdi 1000	Trahan 100	Trahan 200	Trahan 400	Trahan 700	Trahan 1000
Benson	+	+	+	±	±	+	+	×	–	–	±	±	–	–	–	×	–	–	–	–	±	×	–	–	–
Lee	+	±	–	–	–	+	+	+	+	±	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sykes	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–
Laverdi	–	–	–	–	–	×	–	–	–	–	±	×	–	–	–	+	+	+	+	±	–	–	–	–	–
Trahan	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+
Williams	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Masates	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Godfrey	+	+	±	±	–	+	+	±	±	×	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Rampin	–	–	–	–	–	–	–	–	–	–	±	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B. I.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

STRAINS	Williams 100	Williams 200	Williams 400	Williams 700	Williams 1000	Masates 100	Masates 200	Masates 400	Masates 700	Masates 1000	Godfrey 100	Godfrey 200	Godfrey 400	Godfrey 700	Godfrey 1000	Rampin 100	Rampin 200	Rampin 400	Rampin 700	Rampin 1000	B. I. (A)† 100	B. I. (A)† 200	B. I. (A)† 400	B. I. (A)† 700	B. I. (A)† 1000	CONTROLS
Benson	+	±	×	–	–	±	±	×	–	–	±	×	–	–	–	±	±	×	–	–	+	+	±	×	–	–
Lee	–	–	–	–	–	±	×	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	±	±	–	–
Sykes	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Laverdi	–	–	–	–	–	±	±	×	–	–	–	–	–	–	–	×	–	–	–	–	+	±	–	–	–	–
Trahan	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Williams	+	+	+	+	±	–	–	–	–	–	×	–	–	–	–	±	×	–	–	–	+	+	±	×	–	–
Masates	–	–	–	–	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Godfrey	±	±	×	–	–	+	±	±	×	–	+	+	+	±	–	–	–	–	–	–	+	+	±	±	×	–
Rampin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	±	–	–	–	–	–	–	–
B. I.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	–

* Autopsy strains.
† B. I. isolated 1914.
\+ = complete agglutination.
± = strong agglutination.
× = weak or trace agglutination.

was any indication of close relationship or identity, agglutinin absorption was resorted to. The method of absorption was as follows. The growth from twenty-four-hour cultures on "chocolate" medium was scraped into 0.8 per cent NaCl solution and centrifuged until the organisms were sedimented and tightly packed in the tip of the centrifuge tube. Sufficient serum was added so that the volume of the bacilli to the total volume of serum dilution and bacilli would be as 1 is to about 6 to 10. Depending on the volume of bacilli, the amount of serum needed was calculated and sufficient saline was added to give a final dilution of 1–15. To compensate for the water in the packed mass of organisms, the volume was considered as one-half water. This in actuality is an over calculation so that the subsequent dilutions are actually lower than stated. We intentionally adopted this procedure as it gave no advantage to the test, therefore adding to the conclusiveness of the findings.

The organisms were thoroughly distributed in the diluted serum; first by breaking up the clump at the tip of the tube with a platinum loop, then by shaking until an even suspension was secured. The mixtures were incubated in a water bath at 45°C. for three hours and in order to keep the organisms constantly in contact with the serum, the tubes were shaken every fifteen minutes during this period. The mixtures were then stored in the ice-box over night. On the following morning, they were centrifuged and the supernatant fluids were used for agglutination in the requisite dilutions. Control experiments have shown that the absorption mass was three to four times the amount necessary to obtain complete absorption of a serum when the homologous strains were used. It seemed preferable to employ a large absorption dose to bring out any common although quantitatively different absorptive capacity, even though this introduced a possible source of error due to non-specific absorption. Further control experiments since carried out, have shown that the latter does not occur with doses of this size. Thus 2 cc. of serum diluted 1–20 was absorbed with masses of culture ranging from 0.1 cc. to 1.75 cc. of packed culture. The heterologous strain used for these absorptions

gave considerable direct cross agglutination with the serum employed. In spite of this, even the largest dose of this strain did not appreciably diminish the agglutinins for the homologous serum strain.

The results of the absorptions with the strains and sera already described are given in table 2.

The results given in table 2 indicate that the ten strains studied are different one from the other, or in other words from nine cases of influenza were isolated nine distinct races of the influenza bacilli. This result was so surprising that it seemed advisable to extend the study to another series of cultures, especially those isolated in the early stages of the disease; also from cases during convalescence, as well as strains isolated from apparently normal throats. An additional series of cultures obtained post mortem from lung, larynx, or trachea also, six strains isolated by mouse passage, from sputa of suspected influenza pneumonia, were included.

Two antisera prepared with nasopharyngeal strains isolated early in the disease were employed in addition to the nine sera employed in the previous series. The results of these agglutinations as well as the strains employed are given in tables 3 and 3a.

The results in these tables again indicate relatively little relationship between the strains employed and the strains used for the antiserum.

In some instances, a considerable degree of cross agglutination was noted. Te determine again whether this indicated a close relationship or identity, absorption was resorted to.

The results presented in table 4 show that, in two instances, identity in absorptive capacity were encountered. The Sykes post mortem strain was identical with the L.4. nasopharyngeal strain isolated during convalescence. The Laverdi post mortem strain was identical with the mouse 2 strain obtained from sputum by mouse passage. Possible contact between the individuals from whom the first two strains were isolated can be definitely excluded. The other two strains were isolated from sailors admitted to the Willard Parker Hospital from a United States

Receiving Ship within eight days of each other. In this instance, there is a probability of contact. Conclusive data is not available.

Although these two instances of identity were encountered, the general absence of relationship of the strains from this series

TABLE 2*

Agglutinin absorptions

SERUM	AGGLUTINATION BEFORE ABSORPTION							ABSORBED BY	AGGLUTINATION AFTER ABSORPTION									
									Of absorbing strain					Of homologous strain				
	Strains	50	100	200	400	700	Control		50	100	200	400	700	50	100	200	400	700
Benson	Benson	+	+	+	+	+	−	Benson	−	−	−	−	−	−	−	−	−	−
	Lee	+	+	±	−	−	−	Lee	−	−	−	−	−	+	+	+	+	±
	Godfrey	+	+	±	×	−	−	Godfrey	−	−	−	−	−	+	+	+	+	±
Lee	Benson	+	+	+	×	−	−	Benson	−	−	−	−	−	+	+	+	+	±
	Lee	+	+	+	+	+	−	Lee	−	−	−	−	−	−	−	−	−	−
	Godfrey	+	+	+	±	×	−	Godfrey	−	−	−	−	−	+	+	+	+	×
Trahan	Benson	±	±	×	−	−	−	Benson	−	−	−	−	−	+	+	+	+	+
	Trahan	+	+	+	+	+	−	Trahan	−	−	−	−	−	−	−	−	−	−
Williams	Benson	+	±	±	−	−	−	Benson	−	−	−	−	−	+	+	+	+	+
	Williams	+	+	+	+	+	−	Williams	−	−	−	−	−	−	−	−	−	−
	Godfrey	+	±	×	−	−	−	Godfrey	−	−	−	−	−	+	+	+	+	+
Masates	Benson	±	±	×	−	−	−	Benson	−	−	−	−	−	+	+	+	+	+
	Laverdi	±	±	×	−	−	−	Laverdi	−	−	−	−	−	+	+	+	+	+
	Masates	+	+	+	+	+	−	Masates	−	−	−	−	−	−	−	−	−	−
	Godfrey	+	+	±	×	−	−	Godfrey	−	−	−	−	−	+	+	+	+	+
Godfrey	Benson	±	×	−	−	−	−	Benson	−	−	−	−	−	+	+	+	±	±
	Williams	×	−	−	−	−	−	Williams	−	−	−	−	−	+	+	+	+	±
	Godfrey	+	+	+	+	±	−	Godfrey	−	−	−	−	−	−	−	−	−	−
Rampin	Benson	±	±	±	×	−	−	Benson	−	−	−	−	−	+	+	+	+	×
	Williams	±	×	×	−	−	−	Williams	−	−	−	−	−	+	+	+	+	×
	Rampin	+	+	+	+	±	−	Rampin	−	−	−	−	−	−	−	−	−	−

* Autopsy strains. Direct agglutinations in table 1.

STRAINS			AGGLUTINATING SE																													
Group names and numbers	Condition	Period of disease	H II†						M II A†						Benson						L											
			50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	700								
L 3	Influenza?		Not tested	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
L 4	Influenza	Convalescent, 6 weeks		−	−	−	−	−		−	−	−	−	−	×	−	−	−	−	−	−	−	−	−								
L 5	Influenza	Convalescent, 5 weeks		−	−	−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
L 7	Influenza	4 days		±	×	−	−	−		−	−	−	−	−	+	±	×	−	−	−	±	±	×	−								
L 8	Influenza	Convalescent, 6 weeks		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
S 1	Influenza	5 days	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
S 2	Influenza	1 day	+	+	±	±	×	0	−	−	−	−	−	−	−	−	−	−	−	−	±	×	−	−								
S	Influenza	5 days	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
S 4	Influenza	3 days	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
S 5	Influenza	4 days	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	×	−	−								
S 6	Influenza	5 days	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
S 7	Influenza	5 days	×	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
S 8	Influenza	4 days	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
ML 1	Influenza	3 days	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
ML 2	Influenza	3 days	+	±	×	−	−	−	+	+	+	±	−	−	−	−	−	−	−	×	−	−	−	−								
ML 3	Influenza	3 days	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
ML 4	Influenza	3 days	±	×	−	−	−	−	±	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
ML 5	Influenza	3 days	−	−	−	−	−	−	×	−	−	−	−	−	±	−	−	−	−	−	−	−	−	−								
ML 6	Influenza	3 days	×	−	−	−	−	−	×	−	−	−	−	−	±	×	−	−	−	±	×	−	−	−								
Sar	Influenza	3 days	+	±	−	−	−	−	+	±	×	−	−	−	±	×	−	−	−	+	+	±	−	−								
Lit	Influenza	1 day	×	−	−	−	−	−	±	−	−	−	−	−	±	−	−	−	−	±	×	−	−	−								
Hud	Influenza?	Convalescent, 4 weeks	−	−	−	−	−	−	−	−	−	−	−	−	±	×	−	−	−	±	±	×	−	−								
Rec. 10	Influenza	3 days	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
Rec. 11	Influenza	4 days	±	×	−	−	−	−	±	×	−	−	−	−	±	×	−	−	−	×	−	−	−	−								
Han	Influenza	3 days	−	−	−	−	−	−	×	−	−	−	−	−	−	−	−	−	−	×	−	−	−	−								
Grahs	Influenza	3 days	−	−	−	−	−	−	±	×	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
L. lung	Influenza	Autopsy	±	±	−	−	−	−	±	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
trach	Influenza	Autopsy	+	±	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	±	−	−	−	−								
V. lung	Influenza	Autopsy	±	×	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
trach	Influenza	Autopsy	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
C. lung	Influenza	Autopsy	−	−	−	−	−	−	×	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
trach	Influenza	Autopsy	±	×	−	−	−	−	±	×	−	−	−	−	−	−	−	−	−	±	×	−	−	−								
B. trach	Influenza	Autopsy	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	×	−	−	−	−								
D. lung	Influenza	Autopsy	×	−	−	−	−	−	±	×	×	−	−	−	−	−	−	−	−	−	−	−	−	−								
P. trach	Influenza	Autopsy	±	×	−	−	−	−	×	−	−	−	−	−	×	−	−	−	−	−	±	±	×	−								
Mouse 1‡	Inf. pneu.	7 days	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
Mouse 2	Influenza	6 days	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
Mouse 3	Influenza	4 days	×	×	−	−	−	−	±	×	−	−	−	−	±	×	−	−	−	−	−	−	−	−								
Mouse 4	Influenza	4 days	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
Mouse 5	Inf. pneu.	7 days	+	+	±	±	×	0	+	±	−	−	−	−	±	×	−	−	−	±	×	−	−	−								
Mouse 6	Influenza	4 days	−	−	−	−	−	×	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−								
Strains homologous to sera			+	−	+	±	×	−	+	+	+	=	=	−	+	+	+	+	±	±	+	+	−	−								

* General series.

† H II = Hebrew orphan strain—see table 5 a.
M II a = Marine strain—see table 5.

‡ Mouse 1–6 Recovered from mouse after inoculation of sputum.

…nations

…S USED																																							
…			Laverdi						Trahan						Williams						Masates						Godfrey						Rampin						CONTROLS
…00	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Not tested						–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	±	–	–	–	–	–	–	–	–	–	–	–	–	–	Not tested						–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Not tested						–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Not tested						–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Not tested						–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Not tested						–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
[illegible]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Not tested						–	–	–	–	–	–	×	–	–	–	–	–	×	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Not tested						–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Not tested						–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Not tested						±	×	–	–	–	–	–	–	–	–	–	–	±	×	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Not tested						–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Not tested						–	–	–	–	–	–	×	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Not tested						–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Not tested						–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Not tested						+	±	±	×	–	–	–	–	–	–	–	–	±	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Not tested						–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Not tested						–	–	–	–	–	–	–	–	–	–	–	–	±	–	–	–	–	–	–
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Direct agglutin

STRAINS		AGGLUTINATI																									
Group names and numbers	Condition	H II						M II A						Benson													
		50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	50	100
O 1	Normal		−	−	−	−	−		−	−	−	−	−		−	−	−	−	−								
O 2	Normal		−	−	−	−	−		×	−	−	−	−		−	−	−	−	−								×
O 3	Normal		×	−	−	−	−		−	−	−	−	−		−	−	−	−	−								±
O 4	Normal		−	−	−	−	−		−	−	−	−	−		−	−	−	−	−								−
O 5	Normal		×	−	−	−	−		−	−	−	−	−		−	−	−	−	−								−
O 6	Normal		−	−	−	−	−		×	−	−	−	−		−	−	−	−	−								−
O 7	Normal		±	×	−	−	−		+	+	±	±	−		±	−	−	−	−								+
O 8			×	−	−	−	−		+	±	±	−	−		×	−	−	−	−								±
O 9	Normal		−	−	−	−	−		−	−	−	−	−		−	−	−	−	−								−
O 10	Normal		×	−	−	−	−		−	−	−	−	−		−	−	−	−	−								×
O 11	Normal		−	−	−	−	−		×	−	−	−	−		−	−	−	−	−								−
O 12	Normal		±	±	−	−	−		±	×	−	−	−		×	−	−	−	−								−
O 13	Normal		−	−	−	−	−		−	−	−	−	−		−	−	−	−	−								×
O 14			−	−	−	−	−		−	−	−	−	−		−	−	−	−	−								×
O 15			−	−	−	−	−		−	−	−	−	−		×	−	−	−	−								×
O 16			−	−	−	−	−		−	−	−	−	−		−	−	−	−	−								−
O 17	Normal		−	−	−	−	−		−	−	−	−	−		−	−	−	−	−								−
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P 2	Normal	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	−								+
P 3	Normal	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								−
P 4	Normal	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								−
P 5	Influenza†	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								−
P 6	Normal	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								−
P 7	Normal	−	−	−	−	−	−	−	−	−	−	−	−	×	−	−	−	−	−								×
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W 5	Normal		−	−	−	−	−		−	−	−	−	−	±	−	−	−	−	−								+
W 6	Normal		±	×	−	−	−		±	×	−	−	−	±	×	−	−	−	−								×
W 7	Normal		±	×	−	−	−		±	×	−	−	−	×	−	−	−	−	−								+
Strains homologous to sera		+	+	+	±	×	−	+	+	+	±	±	−	+	+	+	+	±	±	+	+							

* General series continued.

† Convalescent, 4 weeks

…nued

…UTIONS USED

[illegible]			Laverdi						Trahan						Williams						Masates						Godfrey						Rampin						CONTROLS
[illegible]	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	
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of cases to the strains used to produce the antisera was strongly suggestive of multiplicity. A possible explanation would be that not one of the serum strains used was an actual primary

TABLE 4*

SERUM	AGGLUTINATION BEFORE ABSORPTION							ABSORBED BY	AGGLUTINATION AFTER ABSORPTION									
									Of absorbing strain					Of homologous strain				
	Strains	50	100	200	400	700	Control		50	100	200	400	700	50	100	200	400	700
H 11	H 11	+	+	+	+	+	−	H 11	−	−	−	−	−	−	−	−	−	−
	S 2	+	+	±	×	×	−	S 2	−	−	−	−	−	+	+	+	±	±
	Mouse 5	+	+	×	−	−	−	Mouse 5	−	−	−	−	−	+	+	+	+	+
Sykes	Sykes	+	+	+	+	±	−	Sykes	−	−	−	−	−	−	−	−	−	−
	L 4	+	+	+	+	±	−	L 4	−	−	−	−	−	−	−	−	−	−
M 11A	M 11A	+	+	+	+	±	−	M 11A	−	−	−	−	−	−	−	−	−	−
	ML 2	+	+	±	−	−	−	ML 2	−	−	−	−	−	+	+	+	=	×
	O 7	+	+	+	±	×	−	O 7	−	−	−	−	−	+	+	+	±	−
Laverdi	Laverdi	+	+	+	+	±	−	Laverdi	−	−	−	−	−	−	−	−	−	−
	Mouse 2	±	±	±	±	±	−	Mouse 2	−	−	−	−	−	−	−	−	−	−
	C. trach.	+	±	±	×	−	−	C. trach.	−	−	−	−	−	+	+	+	±	×
	C. lung	+	±	×	−	−	−	C. lung	−	−	−	−	−	+	+	+	+	×
Godfrey	Godfrey	+	+	+	±	±	−	Godfrey	−	−	−	−	−	−	−	−	−	−
	C. trach.	±	±	×	−	−	−	C. trach.	−	−	−	−	−	+	+	±	×	−
Masates	Masates	+	+	+	+	+	−	Masates	−	−	−	−	−	−	−	−	−	−
	Borham	+	+	+	+	±	−	Borham	−	−	−	−	−	+	+	+	+	+
	Sareska	+	+	±	×	−	−	Sareska	−	−	−	−	−	+	+	+	+	+
Lee	Lee	+	+	+	+	±	−	Lee	−	−	−	−	−	−	−	−	−	−
	Sareska	+	+	±	×	−	−	Sareska	−	−	−	−	−	+	+	+	±	−

* General series. Direct agglutinations in tables 3 and 3a.

infecting epidemic strain but rather a secondary infecting strain of the same species. If *B. influenzae* were the actual primary etiological agent of the disease, the explanation just referred to would seem somewhat remote, in view of the origin of most of

the strains from post-mortem cases which is evidence that the strains possess invasive power. To obtain more direct evidence as to the multiplicity of the strains of *B. influenzae* as well as on the question of the possible occurrence of primary and secondary infecting strains of this microörganism, two further series of cases were investigated.

Two groups were studied, one from a group of marines who had lived for some time in an isolated camp, the other from the inmates of an infant asylum. Two or more isolations were made from a considerable number of the cases. If a definite strain was the primary etiological factor, it would not seem possible to fail to demonstrate a considerable degree of identity in the strains, in such series. Antisera were prepared with four cultures from the marine group and with one culture from the asylum group. The reactions obtained from these sera against the cultures of the same groups are given in tables 5 and 5a.

The cross reactions were no more marked than in the previous series with the exception of one antiserum of the marine group. This serum gave many marked cross reactions, several equalling or exceeding the reactions obtained with the homologous strain.

Agglutinin absorptions were again carried out in the instances where possible relationships were indicated. These results are given in table 6. In the marine group, two strains from different individuals were found to have the same agglutinin absorptive capacity (M. 1 and M. 4). Two strains from different individuals were also found to be identical in the asylum series.

In spite of the marked cross agglutination shown by antiserum M. 7 none of the strains from other individuals absorbed the specific agglutinins of this serum. In other words in spite of close contact no greater proportion of identical strains was encountered in this series than in the general series already described. A noteworthy finding is the result obtained with three successively isolated strains from marine no. 7. As is noted, two earlier isolations were alike, whereas the later isolation was a different type. The successive isolation from marines nos. 1, 3, 4 and 11 are identical. The appearance of different

TABLE 5

Marine series. Direct agglutinations.

STRAINS			AGGLUTINATING SERA AND DILUTIONS USED																							
Numbers	Condition	Period of disease	M 3						M 4						M 7						M 11						Control
			50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	
		days																									
M 1A*	Influenza	4	±	×	−	−	−	−	+	+	+	±	×	−	+	+	+	±	×	−	±	×	−	−	−	−	−
M 1B*	Influenza	6	±	×	−	−	−	−	+	+	+	±	×	−	±	±	±	×	−	−	×	−	−	−	−	−	−
M 2A	Influenza	4	−	−	−	−	−	−	±	×	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M 3A	Influenza	4	+	+	+	±	×	−	±	±	×	−	−	−	+	+	+	±	×	−	−	−	−	−	−	−	−
M 3B	Influenza	8	+	+	+	+	±	−	+	+	±	±	×	−	+	+	+	±	−	−	±	−	−	−	−	−	−
M 3C	Influenza	11	+	+	+	±	×	−	+	±	±	×	−	−	+	+	±	×	−	−	−	−	−	−	−	−	−
M 4A	Influenza	2	±	×	−	−	−	−	+	+	±	×	−	−	+	±	×	−	−	−	±	±	×	−	−	−	−
M 4B	Influenza	5	−	−	−	−	−	−	+	+	+	±	×	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M 5A	Influenza	6	±	×	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	×	×	−	−	−	−	−	−
M 5B	Influenza	10	−	−	−	−	−	−	×	−	−	−	−	−	+	+	±	×	−	−	−	−	−	−	−	−	−
M 6A	Influenza	4	−	−	−	−	−	−	−	−	−	−	−	−	±	×	−	−	−	−	±	×	−	−	−	−	−
M 6B	Influenza	6	±	±	×	−	−	−	−	−	−	−	−	−	±	×	−	−	−	−	+	+	±	±	×	−	−
M 6C	Influenza	8	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M 7A	Influenza	3	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	±	−	−	−	−	−	−	−
M 7B	Influenza	7	+	+	+	±	−	−	×	−	−	−	−	−	+	+	+	±	±	±	×	−	−	−	−	−	−
M 7C	Influenza	14	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−
M 8A	Influenza	4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M 8B	Influenza	7	×	−	−	−	−	−	−	−	−	−	−	−	±	±	±	×	−	−	×	−	−	−	−	−	−
M 9A	Influenza	4	×	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	±	−	−	−	−	−
M 9B	Influenza	7	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	±	×	−	−	−	−
M 10A	Influenza	3	±	±	×	−	−	−	−	−	−	−	−	−	+	+	+	±	×	−	−	−	−	−	−	−	−
M 10B	Influenza	7	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M 10C	Influenza	13	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−
M 11A	Influenza	4	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	×	−	−	+	+	+	+	±	−	−
M 11B	Influenza	8	×	−	−	−	−	−	−	−	−	−	−	−	+	±	×	−	−	−	+	+	+	+	±	×	−
M 11C	Influenza	15	−	−	−	−	−	−	−	−	−	−	−	−	±	±	−	−	−	−	+	+	+	+	+	±	−
M 12A	Influenza	5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M 12B	Influenza	9	±	±	×	−	−	−	−	−	−	−	−	−	+	+	+	±	×	−	±	±	×	−	−	−	−
M 13	Influenza	3	+	+	+	+	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M 14A	Influenza	4	±	×	×	−	−	−	−	−	−	−	−	−	+	+	+	±	×	−	−	−	−	−	−	−	−
M 14B	Influenza	9	−	−	−	−	−	−	±	×	−	−	−	−	+	+	+	±	×	−	±	−	−	−	−	−	−
M 15A	Influenza	4	×	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	×	−	−	−	−	−
M 15B	Influenza	6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−
M 16A	Influenza	4	±	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	±	×	±	×	−	−	−	−	−
M 16B	Influenza	7	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	±	±	×	−	−	−	−	−	−
M 17B	Influenza	9	−	−	−	−	−	−	×	−	−	−	−	−	±	×	−	−	−	−	−	−	−	−	−	−	−
M 18A	Influenza	2	±	×	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	±	±	−	−	−	−

TABLE 5—*Continued*

STRAINS			AGGLUTINATING SERA AND DILUTIONS USED																								
Numbers	Condition	Period of disease	M 3						M 4						M 7						M 11						Control
			50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	
		days																									
M 1SB	Influenza	6	−	−	−	−	−	−	−	−	−	−	−	−	+	±	×	−	−	−	−	−	−	−	−	−	−
M 19A	Influenza		−	−	−	−	−	−	−	−	−	−	−	−	+	±	±	×	−	−	−	−	−	−	−	−	−
M 19B	Influenza		±	±	×	−	−	−	×	−	−	−	−	−	+	+	+	±	×	−	+	±	−	−	−	−	−
M 19C	Influenza		−	−	−	−	−	−	−	−	−	−	−	−	+	±	×	−	−	−	−	−	−	−	−	−	−
M 20A	Influenza	3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	×	−	−	−	−	−
M 20B	Influenza	5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M 21A	Influenza	5	−	−	−	−	−	−	±	±	×	−	−	−	−	−	−	−	−	−	±	×	−	−	−	−	−
M 21B	Influenza	7	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M 21C	Influenza	11	+	±	×	−	−	−	−	−	−	−	−	−	±	×	−	−	−	−	−	−	−	−	−	−	−
M 22	Influenza	4	±	×	−	−	−	−	+	±	×	−	−	−	+	±	×	−	−	−	×	−	−	−	−	−	−
M 26	Influenza	5	−	−	−	−	−	−	±	±	×	−	−	−	±	×	−	−	−	−	×	−	−	−	−	−	−
M 27	Influenza	3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	×	−	−	−	−	−
M 30	Influenza	3	−	−	−	−	−	−	−	−	−	−	−	−	±	±	×	−	−	−	−	−	−	−	−	−	−
M 44	Influenza	2	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	±	×	−	−	−	−	−	−	−	−
M 45	Influenza	3	−	−	−	−	−	−	±	±	×	−	−	−	+	+	+	±	×	−	−	−	−	−	−	−	−

* Letters refer to successive isolations from the same individual.

TABLE 5A

Hebrew orphans. Direct agglutinations

STRAINS			AGGLUTINATING SERUM					
			H II					CONTROL
Number	Condition	Period of disease	100	200	400	700	1000	
H 1	Influenza	1 day	−	−	−	−	−	−
H 2	Influenza	10 days	−	−	−	−	−	−
H 3	Influenza	Early*	−	−	−	−	−	−
H 4	Contact		−	−	−	−	−	−
H 5	Influenza	Early*	−	−	−	−	−	−
H 6	Influenza	5 days	−	−	−	−	−	−
H 7	Influenza	1 day	−	−	−	−	−	−
H 8	Influenza	Early*	±	×	×	−	−	−
H 9	Influenza	1 day	−	−	−	−	−	−
H 10	Influenza	4 days	−	−	−	−	−	−
H 11	Influenza	1 day	+	+	+	±	±	−
H 12	Influenza	1 day	−	−	−	−	−	−
H 13	Influenza	2 days	+	+	+	±	±	−
H 14	Influenza	Early*	−	−	−	−	−	−
H 15	Influenza	3 days	−	−	−	−	−	−
H 16	Influenza	Early*	−	−	−	−	−	−
H 17	Influenza	5 days	−	−	−	−	−	−
H 18	Influenza	Early*	−	−	−	−	−	−
H 19	Influenza	Early*	−	−	−	−	−	−
H 20	Influenza	Early*	−	−	−	−	−	−
H 21	Influenza	Early*	−	−	−	−	−	−
H 22	Influenza	Early*	−	−	−	−	−	−
H 23L†	Influenza	Autopsy	−	−	−	−	−	−
H 24L	Influenza	Autopsy	−	−	−	−	−	−
H 25L	Influenza	Autopsy	−	−	−	−	−	−
H 25T	Influenza	Autopsy	−	−	−	−	−	−
H 27L	Influenza	Autopsy	−	−	−	−	−	−
H 27T	Influenza	Autopsy	−	−	−	−	−	−

* Detailed data not available, early cases 2 to 4 days of disease.

† L = Lung; T = Trachea.

TABLE 6*

SERUM	AGGLUTINATION BEFORE ABSORPTION							ABSORBED BY	AGGLUTINATION AFTER ABSORPTION									
									Of absorbing strain					Of homologous strain				
	Strains	50	100	200	400	700	Control		50	100	200	400	700	50	100	200	400	700
M 3A	M 3A	+	+	+	±	×	−	M 3A	−	−	−	−	−	−	−	−	−	−
	M 3B	+	+	+	+	±	−	M 3B	−	−	−	−	−	−	−	−	−	−
	M 3C	+	+	+	+	±	−	M 3C	−	−	−	−	−	−	−	−	−	−
	M 7B	+	±	×	−	−	−	M 7B	−	−	−	−	−	+	+	+	±	±
	M 13	+	±	±	±	×	−	M 13	−	−	−	−	−	+	+	+	±	±
M 4A	M 1A	+	+	+	±	±	−	M 1A	−	−	−	−	−	−	−	−	−	−
	M 1B	+	+	+	±	±	−	M 1B	−	−	−	−	−	−	−	−	−	−
	M 3B	+	+	+	±	×	−	M 3B	−	−	−	−	−	+	+	+	±	×
	M 4A	+	+	+	+	±	−	M 4A	−	−	−	−	−	−	−	−	−	−
	M 4B	+	+	+	±	×	−	M 4B	−	−	−	−	−	−	−	−	−	−
	M 45	±	±	±	×	−	−	M 45	−	−	−	−	−	+	+	+	±	×
M 7A	M 1A	+	+	+	+	±	−	M 1A	−	−	−	−	−	±	+	+	±	×
	M 3A	+	+	+	±	±	−	M 3A	−	−	−	−	−	±	+	+	±	±
	M 5A	+	+	+	+	±	−	M 5A	−	−	−	−	−	±	±	+	±	×
	M 7A	±	+	+	+	±	−	M 7A	−	−	−	−	−	−	−	−	−	−
	M 7B	+	+	+	+	±	−	M 7B	−	−	−	−	−	−	−	−	−	−
	M 7C	−	−	−	−	−	−	M 7C	−	−	−	−	−	±	+	+	±	±
	M 10A	+	+	+	±	±	−	M 10A	−	−	−	−	−	±	+	+	±	×
	M 11A	+	+	+	+	±	−	M 11A	−	−	−	−	−	±	+	+	±	±
	M 12B	+	+	+	+	±	−	M 12B	−	−	−	−	−	±	+	+	±	×
	M 14A	±	±	×	×	−	−	M 14A	−	−	−	−	−	±	+	+	±	×
	M 14B	+	+	+	±	−	−	M 14B	−	−	−	−	−	±	+	+	±	×
	M 16A	+	+	+	±	±	−	M 16A	−	−	−	−	−	±	+	+	±	±
	M 16B	+	+	+	+	±	−	M 16B	−	−	−	−	−	±	+	+	±	×
	M 19B	+	+	+	+	±	−	M 19B	−	−	−	−	−	±	+	+	±	±
	M 44	+	+	+	±	×	−	M 44	−	−	−	−	−	±	+	+	±	±
	M 45	+	+	+	±	±	−	M 45	−	−	−	−	−	±	+	+	±	±
M 11A	M 11A	+	+	+	±	×	−	M 11A	−	−	−	−	−	−	−	−	−	−
	M 11B	+	+	+	±	×	−	M 11B	−	−	−	−	−	−	−	−	−	−
	M 11C	+	+	+	±	×	−	M 11C	−	−	−	−	−	−	−	−	−	−
H 11	H 11	+	+	+	±	±	−	H 11	−	−	−	−	−	−	−	−	−	−
	H 13	+	+	+	±	±	−	H 13	−	−	−	−	−	−	−	−	−	−

* Marine and Hebrew orphan series. Direct agglutinations in tables 5 and 5a.

TABLE 7*

Strains			Agglutinating sera and dilutions used																																				Controls
Names	Condition	Period of disease	Lorenzo						Michael						Angela						Mary						Gaetano						Caesar						
			50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	50	100	200	400	700	1000	
		days																																					
Lorenzo........	Influenza	1	+	+	+	+	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Michael........	Influenza	5	−	−	−	−	−	−	+	+	+	+	±	±	+	±	×	−	−	−	+	+	+	±	×	−	+	+	±	×	−	−	±	±	×	−	−	−	−
Angela.........	Influenza	5	−	−	−	−	−	−	±	×	−	−	−	−	+	+	+	+	±	±	+	+	+	+	±	×	+	±	±	±	±	×	±	±	±	×	−	−	−
Mary..........	Influenza	5	−	−	−	−	−	−	±	±	×	−	−	−	+	±	×	−	−	−	+	+	+	+	±	×	×	−	−	−	−	−	×	−	−	−	−	−	−
Gaetano.......	Influenza	5	−	−	−	−	−	−	+	+	+	±	×	−	+	+	+	±	×	−	+	+	+	+	±	×	+	+	+	+	+	+	±	±	±	×	×	−	−
Caesar.........	Influenza	3	−	−	−	−	−	−	+	+	±	×	−	−	−	−	−	−	−	−	±	×	−	−	−	−	−	−	−	−	−	−	+	+	+	+	±	×	−

* Family series.

strains in the throat of M. 7 is not necessarily an indication of primary and secondary infection by two strains of the same bacterium. The individual may have possessed the two strains originally, or the second strain may have been implanted by contact.

TABLE 8*

SERUM	AGGLUTINATION BEFORE ABSORPTION								ABSORBED BY STRAINS	AGGLUTINATION AFTER ABSORPTION											
										Of absorbing strain						Of homologous strain					
	Strains	50	100	200	400	700	1000	Control		50	100	200	400	700	1000	50	100	200	400	700	1000
Michael...	Michael	+	+	+	+	±	×	−	Michael	−	−	−	−	−	−	−	−	−	−	−	−
	Gaetano	+	+	+	±	×	−	−	Gaetano	−	−	−	−	−	−	+	+	+	±	×	−
	Caesar	+	+	±	×	−	−	−	Caesar	−	−	−	−	−	−	+	+	+	±	×	−
Angela....	Michael	+	±	×	−	−	−	−	Michael	−	−	−	−	−	−	+	+	+	+	+	±
	Angela	+	+	+	+	+	±	−	Angela	−	−	−	−	−	−	−	−	−	−	−	−
	Mary	+	+	±	×	−	−	−	Mary	−	−	−	−	−	−	+	+	+	+	+	±
	Gaetano	+	+	+	±	×	−	−	Gaetano	−	−	−	−	−	−	+	+	+	+	+	±
Mary.....	Michael	+	+	+	+	±	×	−	Michael	−	−	−	−	−	−	+	+	+	±	×	−
	Angela	+	+	+	+	±	×	−	Angelo	−	−	−	−	−	−	+	+	+	±	×	−
	Mary	+	+	+	+	±	×	−	Mary	−	−	−	−	−	−	−	−	−	−	−	−
	Gaetano	+	+	+	+	±	×	−	Gaetano	−	−	−	−	−	−	+	+	+	±	×	−
Gaetano...	Michael	+	+	±	×	−	−	−	Michael	−	−	−	−	−	−	+	+	+	+	+	±
	Angela	±	±	±	×	−	−	−	Angela	−	−	−	−	−	−	+	+	+	+	+	±
	Gaetano	+	+	+	+	+	±	−	Gaetano	−	−	−	−	−	−	−	−	−	−	−	−
Caesar....	Michael	+	±	±	±	−	−	−	Michael	−	−	−	−	−	−	+	+	+	+	±	×
	Angela	±	±	×	×	−	−	−	Angela	−	−	−	−	−	−	+	+	+	+	±	×
	Gaetano	±	±	×	−	−	−	−	Gaetano	−	−	−	−	−	−	+	+	+	+	±	×
	Caesar	+	+	+	+	±	×	−	Caesar	−	−	−	−	−	−	−	−	−	−	−	−

* Family series. Direct agglutinations in table 7.

An opportunity arose to study the strains isolated from the members of a single family all ill with influenza. The onset of the disease in the different members was very close together.

Antisera were prepared with the strain isolated from each individual. The results of the direct agglutination and of the agglutinin absorption are given in tables 7 and 8. No identities

were encountered. It would seem that in such a series infected from a common source or one from the other, identities should be encountered, if *B. influenzae* were the primary infectious agent.

TABLE 9

Summary of Investigation

SERIES	NUMBER OF STRAINS TESTED	NUMBER OF ANTISERA FOR GROUP	NUMBER OF OTHER ANTISERA	FINDINGS
Autopsy.....	10	10	7	All strains found to be distinct races. One autopsy strain identical with a miscellaneous strain. Another autopsy strain identical with another miscellaneous strain.
Miscellaneous.....	73	0	18	No two strains were found to be identical. For identities see Autopsy Series above.
Marines.....	54	4	14	Two strains from different individuals were found identical. Of isolations from the same individual. 4th day identical with 6th day 4th day identical with 8th day identical with 11th day 2nd day identical with 8th day 4th day identical with 8th day identical with 15th day 3rd day identical with 7th day, but 14th day was different
Hebrew orphans...	28	5*	16	Two strains were found identical.
Family.....	6	6	10	All strains were found to be distinct races.

* The results with one serum is given in table 5a, but the strains were tested with four other sera. No additional identities were discovered.

For economy of tabulations and to avoid confusion, a considerable amount of agglutination results as well as absorptions are not recorded in the previous tables. Both the Hebrew Infant Asylum and the marine culture series were tested with nearly all the antisera. No identities were discovered in these

tests. Likewise, a marine antiserum other than the one given in tables 3 and 3a was utilized to test nearly all the strains. Here, again, no identities were encountered.

Finally, all the strains still available were tested with antisera, Sykes, Laverdi, Benson, marine no. 4 and Hebrew Infant Asylum no. 11. These were the sera with which the identities were found as noted in the previous tables. Wherever cross-agglutinations were encountered, the results were again checked by agglutinin absorption. No further identities were found.

A summary of the results given in the preceding tables as well as the additional agglutinations above described, is presented in table 9.

DISCUSSION

The results recorded indicate that, under the term *B. influenzae*, we are dealing with a group of organisms which, for practical purposes, is heterogeneous in character as determined by immunological reactions. We have evidences of the existence of small sub-groups. The actual frequency with which identical strains would be encountered could only be determined by the use of many more sera than we have employed. Our own results, however, indicate at least that identical strains would be infrequently encountered.

The existence of a multiplicity of races is advanced as evidence that *B. influenzae* is not the primary etiological agent in epidemic influenza. To controvert this evidence, it would be necessary to prove either that the agglutinin absorption capacity of *B. influenzae* was a characteristic susceptible to rapid change or that, in no instance, have we utilized an actual primary infecting strain for the production of our serums. By analogy with the constancy of the agglutinin absorptive capacity shown by other varieties of bacteria, the former assumption is extremely improbable. The latter assumption that we have failed to employ an actual epidemic strain is rendered very remote especially by the employment of strains isolated early in the disease from groups of cases in close contact.

STUDIES IN PROTEIN INTOXICATION

IV. HISTOLOGIC LESIONS PRODUCED BY INJECTIONS OF PEPTON

T. HARRIS BOUGHTON

From the Department of Pathology of the University of Illinois, College of Medicine, Chicago

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It has long been known that the parenteral injection of albumoses or "pepton" into animals produces marked effects on blood pressure and on the coagulability of the blood. The earliest important work was reported by Schmidt-Muelheim in 1880 (1) and by Fano in 1881 (2). The similarity of this "pepton poisoning" to anaphylactic shock was noted by de Waele in 1907 (3), by Biedl and Kraus in 1909 (4), and by Arthus (5). Since these original observations were recorded an enormous amount of study has been devoted to this topic with the purpose of analysing the mechanism of this toxic action, and possibly of throwing some light on the mechanism of anaphylaxis. Hardly any observations, however, have been recorded on the histologic changes resulting from pepton poisoning.

Simonds (6) describes marked passive hyperemia occurring in the livers of dogs dying from acute pepton shock, and thrombi in the sinusoids of the livers after several injections of peptone. Nolf (7) mentions damage to the endothelium of hepatic capillaries. A few writers have described the histologic changes occurring in anaphylaxis, but no attempt has been made to compare the effects on the organs and tissues produced by anaphylaxis and by pepton poisoning. The purpose of the present investigation is to make such a comparison. The work on the physiological effects produced by pepton and by purified albumoses when introduced parenterally into various animals has

shown beyond question the very close parallelism between this and the intoxication of sensitized animals by native proteins. DeKruif and Eggerth (8), however, believe that the two phenomena are not identical since they state that the toxic principle of Witte's pepton easily passes membranes that hold back all of the anaphylotoxin. The histologic method of study while not so direct as the physiologic or chemical may yet elicit information not otherwise obtainable.

MATERIAL AND METHODS

In the present study fourteen guinea-pigs were used, varying in weight from 200 to 400 grams. They were injected with Witte's pepton intraperitoneally. The initial dose was usually 0.6 gram per kilo and this amount was increased to the limit of tolerance, which was usually found to be about 2 grams per kilo when the doses were given a few days apart; however, some of the animals resisted several doses of this size. The maximum number of doses received by any animal was eight and the average was five. Death usually occurred several hours after the last injection, but sometimes not until a few days later. The animals were under observation up to seven weeks, and the average length of the observation time was three weeks.

RESULTS

The symptoms usually shown consisted of ruffing of the hair of the head and neck with respiratory distress (frequently hiccough) and prostration. Most commonly if an animal does not succumb to the first injection, a later injection of the same size is without evident effect, but a larger dose will bring on the symptoms noted. On post mortem examination the lungs were found to be fully expanded, and hyperemia of the organs was marked. In a few cases subserous hemorrhages were observed, but these were not marked nor extensive.

On microscopic examination the kidneys showed the most marked change. The epithelium was affected in every case. In most of the sections there was marked and extensive swelling

and vacuolation of the epithelium of the convoluted tubules and of the ascending limb of Henle's loop. In some cases the process went on to complete disruption and necrosis of some of the epithelial cells, and was followed by regeneration. In a few cases vacuolation was not so prominent, and the process proceeded from parenchymatous degeneration to necrosis. The epithelial changes were most marked in those cases in which death occurred soon after the last injection. In cases where several days elapsed before the animal's death the changes noted were somewhat less marked. Hyperemia was present and marked in every case. Small hemorrhages were observed in about one-half of the cases. The vessels of the kidney were almost unaffected: in two cases the walls of the smaller arteries showed slight swelling. There were no areas of fibrosis or infiltration observable.

In the livers the epithelium was affected in all but one case, and this was an animal that had received but a single injection. Parenchymatous degeneration was observed in nearly all cases, and was frequently severe. Fatty degeneration of mild grade was also observed. Small areas of focal necrosis were seen in half of the series, and there was some evidence of epithelial regeneration. Leukocytic infiltration was frequently observed, especially about the vessels. The vessels of the liver were markedly altered. Swelling of the walls of the smaller arteries was present in nearly every case. This swelling involved both intima and media, frequently rendering the internal elastic lamina unduly prominent, and occasionally showing fissuring of the media. In most of the sections proliferation of the endothelial cells of the intima could be seen, as evidenced by the increased number of nuclei present. Fibrosis was not observed.

In the hearts the only pathological change noted was round celled infiltration which, however, was present in practically all cases, and was marked in a few.

The gross appearance of the organs post mortem, and the lesions found microscopically in this series show a striking similarity to those found in guinea-pigs with anaphylaxis (9). In general the lesions produced are somewhat less severe in the

pepton animals. This may be due to the fact that the pepton animals were under observation for a much shorter period than the albumen animals, and received a smaller number of injections. The marked resemblance between the gross and microscopic changes produced by injections of pepton and by injections of native proteins (especially when considered in connection with the physiological observations recorded by many authors) furnishes another reason for considering these two phenomena as closely related.

CONCLUSIONS

1. Intraperitoneal injections of Witte's pepton into guinea-pigs produce lesions of the liver, heart, and kidney.

2. These lesions consist of degeneration and necrosis of epithelium, followed by regeneration; of edema of the walls of the smaller arteries, with endothelial proliferation; of perivascular infiltration; and of hyperemia.

3. On post mortem examination the lungs are expanded and the organs hyperemic.

4. Both gross and microscopic lesions are very similar to those produced in guinea-pigs by injections of native proteins.

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OBSERVATIONS ON THE PRODUCTION OF AN ANTI-HAEMOTOXIN FOR THE HAEMOTOXIN OF BACTERIUM WELCHII (BACILLUS AEROGENES CAPSULATUS)

WILLIAM W. FORD AND GEORGE HUNTINGTON WILLIAMS

From the Department of Bacteriology, School of Hygiene and Public Health, Johns Hopkins University

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It has previously been shown by Ford and Lawrence (1) that the hemolytic property of milk cultures of *Bacterium welchii* is to be attributed to the production of a true bacterial haemolysin or haemotoxin by this organism. The opinion previously held that the destruction of blood corpuscles in cultures of this species is due to the presence of acids (butyric and lactic) is not justified, since milk cultures retain their blood-laking powers after complete neutralisation. In tests with the neutralised whey the discoloration of the corpuscles due to the production of methaemoglobin from acids is not seen and the haemolysed blood takes on the brilliant red color usual with true bacterial haemotoxins. Furthermore, cultures of the gas bacillus lose their haemolytic activity on being heated to 62–63°C., while solutions of both butyric and lactic acids can be boiled some time without losing their power to destroy blood corpuscles. Finally, the haemolysin of *B. welchii* is digested by pepsin-HCl and by pancreatin, and is precipitable with ethyl alcohol, properties which also tend to place this substance in the group of bacterial secretory products. During the course of the past few months the study of the haemotoxin of the gas bacillus has been continued and our previous observations have been confirmed. In addition several new facts have been brought out which it seems desirable to report at the present time.

SOURCE OF THE CULTURES

The strains of *B. welchii* which have been employed for the study of the haemolysins found in milk cultures have been obtained in all instances from samples of Baltimore milk. Quantities varying from 500 to 1000 cc. have been heated to temperatures ranging from 80° to 85°C. for twenty to th rty minutes and incubated at 37°C. for twenty-four to forty-eight hours. In the majority of such samples the characteristic reaction of stormy fermentation appears after the lapse of this time. Such milk cultures contain aerobic bacteria and a number of other anaerobes in addition to the gas bacillus. Various methods of obtaining pure culture have been tried out. In the early part of the work rabbits were inoculated intravenously with small quantities (1 to 2 cc.), killed in five minutes and kept in a warm place for eighteen to twenty hours after which milk and agar cultures were made from the blood and organs. Frequently other organisms survived in the animal body and appeared in the cultures so that it was necessary to resort to the use of blood-agar plates from which the typical haemolytic colonies could be fished. As the work progressed it was found that this plating in agar could be dispensed with and pure cultures could be obtained from the animals by the simple method of "continuous transfer" of fairly large quantites of culture (1 to 2 cc.) from one milk tube to another. The rapidity with which *B. welchii* develops in milk at 37°C. enables it to overgrow nearly all other species and if transfers are made at the end of every twelve hours some half a dozen transfers suffice to give an uncontaminated strain. Cultures obtained with this apparently crude method have been tested repeatedly. They contain nothing but non-motile encapsulated organisms—Gram-positive with an admixture of Gram-negative forms—which give characteristic reactions in culture media and produce the typical lesions on animal inoculation. Blood-agar plates made from such cultures reveal nothing but haemolytic colonies and aerobic cultures remain steril. We feel confident, therefore, that pure cultures can be obtained easily with this method. The same method of con-

tinuous transfer can be applied to the original milk flasks which have shown the stormy fermentation and in about two-thirds of the samples a pure culture of the gas bacillus can be obtained. In a certain number however the gas bacillus is overgrown by certain acid-resistant streptococci which may happen to survive the original heating of the milk. Animal passage, therefore, is usually advisable. It may be noted that the gas bacillus develops in milk tubes without incubation under anaerobic conditions provided cream is left in the milk to form a surface layer. While this cream layer may be more pervious to oxygen than was previously supposed and does not provide anaerobic conditions in the depths of the medium its presence in the milk has proved a distinctly favorable factor for the cultivation of the gas bacillus. Finally, the difficulty of keeping milk cultures alive may be lessened by the addition of a few particles of powdered calcium carbonate to the milk tubes. The acidity of the cultures is much lessened by this procedure and transfers can be obtained from such tubes twelve to fourteen days old. In general the gas bacillus dies out in milk in three to four days.

PREPARATION OF THE HAEMOTOXIN

The haemotoxin of *Bacterium welchii* can be obtained from massive milk cultures of the organism with the following method. Large flasks of steril milk containing 800 to 1000 cc. are inoculated by pouring into them the contents of a twenty-four to forty-eight hour milk culture (10 to 12 cc.). These flasks are incubated at 37°C. and by the end of twenty-four to forty-eight hours the characteristic reaction usually appears. This material is now filtered through coarse filter paper to remove the particles of curd and the filtrate as it appears is immediately neutralised by caustic soda or caustic potash. The filtration is usually slow, requiring eighteen to twenty hours, during which time there is a further development of organisms in the filtrate with some increase of acidity. The material is again completely neutralised, upon which a thick gelatinous precipitate appears. This settles to the bottom of the flasks and is easily removed by passing the

fluid through filter paper. If this product is not entirely free from bacteria it can now be filtered through a Berkefeld candle. The final solution is clear, straw-colored, somewhat viscid and foams easily on shaking. About 400 cc. of this product can be obtained from a liter culture of the gas bacillus.

STRENGTH OF THE HAEMOTOXIN

Haemolytic solutions prepared by the method here described have a fairly uniform strength. When titrated against rabbits' corpuscles 1 cc. of a 5 per cent suspension of blood cells is usually dissolved completely by 0.05 cc. representing a dilution of 1–20

TABLE 1

Determination of the strength of haemotoxin from neutralised milk cultures of Bacterium welchii

WHEY	RABBIT BLOOD, 5 PER CENT	HEMOLYSIS
cc.	*cc.*	
1.0	1	Complete
0.75	1	Complete
0.5	1	Complete
0.25	1	Complete
0.1	1	Complete
0.075	1	Complete
0.05	1	Complete
0.025	1	Complete
0.01	1	Partial
Control 1 cc. NaCl 0.75 per cent	1	Negative

Strength, 0.025; dilution, 1–40.

of the original material. The reaction takes place somewhat slowly, requiring at least four hours at 37°C. In some instances the haemolysin is stronger than this, the haemolytic unit being 0.025 cc., a dilution of 1–40, but this is somewhat uncommon. Very rarely a more powerful haemotoxin is found with a haemolytic unit of 0.01 cc., a dilution of 1–100.

STABILITY OF THE HAEMOTOXIN

The haemotoxin of the gas bacillus is a relatively stabile substance. When kept in the dark at a low temperature the strength of the solution remains fairly constant. Thus a prep-

aration was made about June 15 of the past year which had a strength of 0.025 cc. (1–40). This was used repeatedly for testing sera for about four weeks during which time it slowly deteriorated until its strength on July 10 was 0.05, a dilution of 1–20. This preparation was then put aside and not tested again till October 26 when it had a strength of 0.075, a dilution of about 1–13. A similar preparation with a strength of 0.2 cc. (1–5) on December 14, 1918, was tested repeatedly for about five months and on May 7, 1919, its strength was the same.

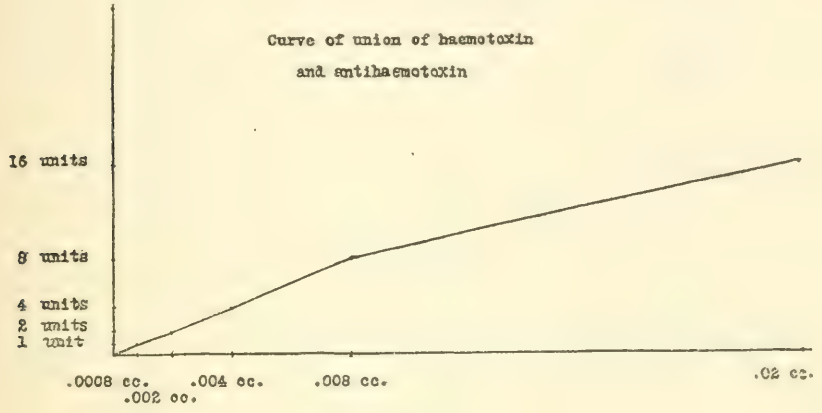

FIG. 1

PRODUCTION OF THE ANTIHAEMOTOXIN

The attempt was now made to immunize rabbits with the haemotoxin of the gas bacillus produced by the method described. Six rabbits were given increasing doses of haemotoxin subcutaneously, starting with small quantities representing low multiples of the haemolytic unit. No definite effect upon the animal's health could be noted. The weight remained stationary and there were no changes at the point of inoculation. As the doses became larger the health of the animals was not appreciably affected but a good deal of subcutaneous oedema and induration developed at the site of inoculation. This oedema and swelling were transient however and they usually disappeared in three or

four days. Of the animals originally selected one died of a laboratory infection and one developed subcutaneous abscesses necessitating its destruction. The other rabbits remained under treatment four months and showed the presence in the blood stream of antihaemolytic substances which could be demonstrated in high dilutions of the serum.

STRENGTH OF THE ANTIHAEMOTOXIN

The serum of rabbits treated with increasing doses of the haemotoxin of the gas bacillus contains an antihaemotoxin for this substance. If the serum be allowed to come in contact with the haemotoxin it neutralises it completely so that when blood corpuscles are subsequently added no solution takes place. The usual strength of the antihaemotoxin in animals under treatment was about 1–1000 when tested against freshly prepared haemolysins with the use of the haemolytic unit above described as the index. This is shown in the following table.

The immune sera were always tested in quantities of 0.1 cc. or less. Normal rabbits' serum in larger amounts, 1 cc., 0.5 cc. and 0.125 cc., protects the corpuscles against several multiples of a haemolytic unit, but this reaction appears to depend upon the bathing of the corpuscles in a protective medium rather than upon a true neutralisation of the haemotoxin. The protection afforded by normal serum never appears beyond a dilution of 1–10. In the serum outlined in table 2 the corpuscles were completely protected against the haemotoxin by a quantity of 0.0008 cc. which represents a dilution of the serum of about 1–1250. The usual strength of the anti-serum was somewhat less, about 1–1000.

METHOD OF COMBINATION

While the titration of the anti-serum against minimal doses of the haemotoxin offers a simple method of determining its strength this method is subject to certain possible errors. The length of time required for solution of the corpuscles by the haemotoxin and for estimating the strength of the antihaemotoxin is too long

and in addition the deterioration of the haemotoxin may be such as to make the quantity regarded as the unit incapable of causing a complete solution of the cells. Later, therefore, the sera were always titrated against several multiples of a haemolytic unit, usually against multiples representing 2, 4, 8 and 16 units. Such a series can be read at the end of four hours and in addition it gives some information as to the mode of union of

TABLE 2

Strength of anti-haemotoxin
Serum from rabbit I tested vs. an haemotoxin of unit strength 0.1

HEMOTOXIN	SERUM	PROTECTION
	cc.	
0.1	0.1	+++
0.1	0.08	+++
0.1	0.06	+++
0.1	0.04	+++
0.1	0.02	+++
0.1	0.01	+++
0.1	0.008	+++
0.1	0.006	+++
0.1	0.004	+++
0.1	0.002	+++
0.1	0.001	+++
0.1	0.0008	+++
0.1	0.0006	++
0.1	0.0004	++
0.1	0.0002	0
0.1	0.0001	0
0.1	1 cc. NaCl 0.75 per cent	0

Complete protection is indicated by +++; partial by ++; slight by +; none by 0.

the antihaemotoxin with the haemotoxin. With fresh preparations the substances combine apparently according to the laws of multiple proportions. This is shown in table 3.

It is interesting to note in this connection that the antihaemolysin produced in animals by the immunization with the haemolysin in fungi also combines with the haemolysin according to the laws of simple multiples, as was shown by Ford and Rockwood (2).

COMBINATION OF ANTISERUM WITH OLD HAEMOTOXIN

As the haemotoxin deteriorates it loses its strength and its method of combination with the antihaemotoxin does not appear to be as simple as when fresh. Larger quantities of anti-serum are required to neutralise given quantities of haemotoxin and the line of union with multiples of a haemolytic unit is no longer a straight line but a rapidly ascending curve. The laws governing this union have not been worked out completely but it apparently follows the same rule as other toxins and antitoxins and may be explained by the theory that toxoids are formed as the haemotoxic solutions age. This will be taken up in a subsequent publication.

TABLE 3

Strength of antihaemotoxin from rabbit I determined by titrating the serum against multiples of a haemotoxic unit

HEMATOXIN		QUANTITY OF SERUM PROTECTING COMPLETELY
UNITS	WHEY	
	cc.	*cc.*
16	1.6	0.02
8	0.8	0.008
4	0.4	0.004
2	0.2	0.002
1	0.1	0.0008

If this series is put in the form of a curve with abscissas and ordinates it will be seen that the union is represented by practically a straight line (see chart 1)

CONCLUSION

By the immunisation of animals with the haemolytic or haemotoxic substance produced in milk by *Bacterium welchii* an anti-haemolysin or anti-haemotoxin of a strength of 1–1000 to 1–1250 has now been produced. The demonstration that this substance can act as an antigen offers the final proof that it belongs to the group of true bacterial haemolysins or true haemotoxins.

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THE INFLUENCE OF DESICCATION UPON NATURAL HEMOLYSINS AND HEMAGGLUTININS IN HUMAN SERA

JOHN A. KOLMER

From the Dermatological Research Laboratories of Philadelphia and the McManes Laboratory of Experimental Pathology of the University of Pennsylvania

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As part of a series of studies upon natural hemolysins and hemagglutinins in human sera (1), the influence of desiccation upon these substances has been investigated and the results have proven of particular interest in reference to the method of Sanford (2) of using normal human sera dried on cover glasses for the purpose of conducting microscopical agglutination test in the classification or typing of blood; these studies have proven especially interesting and of practical importance since Sands and West (3) have found in these laboratories, that drying rabbit antihuman serum in paper and dishes results in the destruction or inactivation of a portion of the immune hemagglutinins and suggesting that the practice of using dried human sera for agglutination tests after the methods of Sanford (2) and Hartman (4), may be open to error due to deterioration of the normal isoagglutinins.

The influence of desiccation upon normal isohemolysins in human sera has also been the subject of investigation; in the studies previously referred to (1) sera were occasionally encountered containing natural hemolysins but no agglutinins for the corresponding corpuscles and I am of the opinion that best results in the matching of bloods for transfusion are obtained by conducting both agglutination and hemolysins tests. It is possible that these hemolysins may produce reactions following the transfusion of blood compatible for the recipient insofar as the agglutinins are concerned.

INFLUENCE OF DESICCATION UPON NATURAL HEMAGGLUTININS

Fresh unheated human sera were examined for the presence of agglutinins by a macroscopic test consisting in mixing in small test tubes 0.1 cc. of each serum with 1 cc. of a 1 per cent suspension of washed corpuscles, incubating at 38°C. in a water bath for an hour and reading the results after the mixtures have stood in a refrigerator over night. Sera showing the presence of hemagglutinins were then dried by distributing 1 cc. of each evenly and carefully over accurately measured squares (usually 60 by 60 mm.) of paper (W. and R. Balston No. 2) and drying them at room temperature; each square was then ruled and cut into ten parts, each part carrying the equivalent of 0.1 cc of serum. Macroscopical agglutination tests were then repeated with each fluid and dried serum at the same time, with the same corpuscles and under identical conditions.

In some experiments the dried sera were kept at room temperature and in a refrigerator while the corresponding fluid sera were kept in a refrigerator over a period of two weeks for the purpose of studying the deterioration of the hemagglutinins under these conditions; since a variation in results could occur with different lots of corpuscles, the same corpuscles were kept in 1:800 formalin in physiological saline (5) over the entire period of observation.

The results of several experiments in which this macroscopic technic was employed are shown in tables 1, 2 and 3; it is to be emphasized that these observations were made macroscopically inasmuch as comparative microscopical and macroscopical tests have shown that the former may show some agglutination which cannot be detected by the naked eye in test tubes. By using 0.1 cc. serum with 1 cc. of 1 per cent suspensions of corpuscles the macroscopical tests were, however, usually clear and decisive and the presence or absence of agglutination was readily determined by comparison with the corpuscle controls.

The reactions shown in table 1 were conducted with the corpuscles of six different persons and the dried sera were employed about three days after preparation; as shown in this table not

all of the isohemagglutinins in human sera are destroyed by drying, but some deterioration usually occurs. A few tests of this kind with typed bloods in which type II and type III sera dried in paper were employed, have shown that the resistance of these isohemagglutinins to drying are about the same and that the disappearance of hemagglutinin for one lot of corpuscles and the persistence of a second is due largely to quantitative relations, titration tests having shown that the latter is present

TABLE 1

The influence of drying serum in paper upon human isoagglutinins

SERUM	RESULTS WITH SERA BEFORE DRYING						RESULTS WITH SERA AFTER DRYING*					
	Corpuscles 1	Corpuscles 2	Corpuscles 3	Corpuscles 4	Corpuscles 5	Corpuscles 6	Corpuscles 1	Corpuscles 2	Corpuscles 3	Corpuscles 4	Corpuscles 5	Corpuscles 6
2	−†	−	±	−	−	+	−	−	−	−	−	−
3	+	−	−	−	+	−	+	−	−	−	+	−
4	+	−	−	+	+	+	−	−	−	−	−	−
8	+	+	−	+	+	+	+	−	−	−	−	+
9	+	−	−	−	−	−	−	−	−	−	−	−
10	−	−	−	−	−	−	−	−	−	−	−	−
11	+	−	−	−	−	−	−	−	−	−	−	−
12	+	−	−	−	+	−	+	−	−	−	+	−
13	+	−	−	−	−	−	−	−	−	−	−	−
15	+	−	−	−	−	−	−	−	−	−	−	−
19	−	−	−	+	−	+	−	−	−	−	−	+
20	+	−	−	−	+	−	+	−	−	−	−	−

* Tested seventy-two hours after drying; kept at room temperature.

† − = no agglutination; ± = partial agglutination; + = agglutination.

in the original serum in larger quantity and consequently present in sufficient amounts in the dried state to produce agglutination despite deterioration of a portion.

Human sera generally contain relatively large amounts of agglutinin for rabbit corpuscles as may be determined by titrations; consequently drying and testing 0.1 cc. of serum may not show much depreciation in agglutinin as shown in table 2; a serum containing a small amount of agglutinin in the fresh state may, however, fail to agglutinate after drying.

Human sera usually contain smaller amounts of agglutinin for rat corpuscles and consequently the influence of drying sera upon these agglutinins is quite apparent; as shown in table 3 considerable deterioration generally occurs.

TABLE 2

The influence of drying human serum in paper upon natural antirabbit agglutinins

SERUM	RESULTS WITH SERA BEFORE DRYING						RESULTS WITH SERA AFTER DRYING*					
	Corpuscles 1	Corpuscles 2	Corpuscles 3	Corpuscles 4	Corpuscles 5	Corpuscles 6	Corpuscles 1	Corpuscles 2	Corpuscles 3	Corpuscles 4	Corpuscles 5	Corpuscles 6
2	+†	+	+	+	+	+	+	+	−	−	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	−	−	+	+	+	−	−	−	+	−
9	+	−	−	−	−	+	−	−	−	−	−	−
10	−	−	−	−	−	−	−	−	−	−	−	−
11	+	+	+	+	−	−	+	−	−	−	−	−
12	+	+	+	+	+	+	+	+	+	+	+	+
17	+	+	+	+	+	+	+	+	+	+	−	−

* Tested ninety-six hours after drying; kept at room temperature.
† + = agglutination; − = no agglutination.

TABLE 3

The influence of drying human serum in paper upon natural antirat agglutinin

SERA	RESULTS WITH SERA BEFORE DRYING						RESULTS WITH SERA AFTER DRYING*					
	Corpuscles 1	Corpuscles 2	Corpuscles 3	Corpuscles 4	Corpuscles 5	Corpuscles 6	Corpuscles 1	Corpuscles 2	Corpuscles 3	Corpuscles 4	Corpuscles 5	Corpuscles 6
3	−†	+	−	+	+	+	−	−	−	−	−	−
8	+	+	+	+	+	+	+	+	−	−	−	−
15	−	−	+	+	+	+	−	−	−	+	+	+
16	+	+	−	+	−	−	−	−	−	−	−	−
20	+	+	+	+	+	+	−	+	+	+	+	−

* Tested seventy-two hours after drying; kept in a refrigerator.
† − = no agglutination; + = agglutination.

Tables 2 and 3 also show the presence of group hemagglutinins in human sera for rabbit and rat corpuscles similar to the group for human corpuscles, previously described (1).

Microscopical tests were conducted by drying sera upon clean cover glasses (two loopfuls of serum placed in the center of a

glass) and allowing them to dry at room temperature; when used, a loopful of a 1 per cent suspension of washed corpuscles was added to the dried serum and thoroughly rubbed up with it and the mixture was suspended in a vaselined hollow ground slide followed by microscopical examination after standing about fifteen minutes at room temperature.

The results of two experiments with anithuman and antirabbit agglutinins in human sera are shown in tables 4 and 5; numerous tests were made over a period of fourteen days to determine whether the agglutinins in dried sera tend to further deterioration.

TABLE 4

The influence of drying human sera on cover glasses upon normal isoagglutinins

SERA	CORPUSCLES	RESULTS WITH FRESH FLUID SERA	RESULTS WITH DRIED SERA*			
			1 day	3 days	7 days	14 days
Type II	Type III	++†	+	+	−	−
Type II	Type III	++	++	++	++	++
Type II	Type III	++	++	+	+	−
Type II	Type III	++	++	++	−	−
Type II	Type I	++	+	+	+	+
Type III	Type II	++	++	++	++	+
Type III	Type II	+	−	−	−	−
Type III	Type II	++	+	−	−	−
Type III	Type II	++	++	++	+	+
Type III	Type I	++	+	+	+	+

* Kept in a refrigerator.

† + + = strong agglutination; + = weak agglutination; − − no agglutination.

As shown in table 4 if a serum contains a large amount of agglutinin capable of producing agglutination of practically all cells in the preparation (+ +), it may show a weaker reaction (+) after drying, some clumps of cells being seen but also large numbers of non-agglutinated corpuscles; a serum yielding a weak reaction in the wet state has been observed to fail to agglutinate after drying. Not infrequently however, sera yielding strong reactions in the fresh state yielded well marked agglutination over a period of two weeks, the dried sera on cover glasses being kept sealed in papers in a refrigerator as described by Sanford. As shown in table 4, deterioration of agglutinins was

found within three or four days after drying; agglutinins escaping destruction in this time were usually preserved in a refrigerator over the two weeks' period of observation.

Similar results were observed with the agglutinins for rabbit corpuscles in human sera (table 5); owing to the presence of these agglutinins in large amounts in human sera, the majority of dried sera showed no appreciable differences in agglutination from the corresponding fluid sera, inasmuch as sufficient agglutinin remained after drying to mask deterioration of a portion of the agglutinins.

TABLE 5

The influence of drying human serum on cover glasses upon natural antirabbit agglutinin

SERA	SERA BEFORE DRYING	SERA AFTER DRYING*				
		1 day	3 days	7 days	14 days	21 days
2	++†	++	++	++	++	++
3	+	+	+	+	+	+
4	++	++	+	+	+	+
6	++	++	++	++	++	++
7	++	+	+	+	+	+
10	++	++	++	++	++	++

* Dried sera kept in a refrigerator.

† + + = strong agglutination; + = weak agglutination.

THE INFLUENCE OF DESICCATION UPON NATURAL HEMOLYSINS

The natural hemolysins in human sera vary in resistance to the deterioration of desiccation; antisheep and antiox hemolysins and especially the former, are most resistant while antihuman, antiguinea-pig and other hemolysins are quite susceptible. With the exception of the natural hemolysin for sheep corpuscles it would appear that the balance of hemolysins present in human sera for human corpuscles and the corpuscles of the lower animals, are more susceptible to deterioration by drying than the corresponding agglutinins.

Experiments with the hemolysins were conducted in the same manner as the macroscopic agglutination tests except that sera about three days old and practically free of complement were

employed in the fluid state and after being dried in paper, complement being furnished to the fluid and dried sera by adding 0.2 cc. of 1:10 dilutions of the mixed sera of guinea-pigs previously absorbed at a low temperature with the corpuscles being used to remove the corresponding natural hemolysin if present.

The results of an experiment with antihuman hemolysins are shown in table 6; the dried sera were employed twenty-four hours after preparation and the results of this and similar experiments show the marked deterioration of these isohemolysins following desiccation of the sera.

TABLE 6

The influence of drying human serum in paper upon normal isohemolysins

SERA	RESULTS WITH SERA BEFORE DRYING						RESULTS WITH SERA AFTER DRYING*					
	Corpuscles 1	Corpuscles 2	Corpuscles 3	Corpuscles 4	Corpuscles 5	Corpuscles 6	Corpuscles 1	Corpuscles 2	Corpuscles 3	Corpuscles 4	Corpuscles 5	Corpuscles 6
1	N†	S	N	S	C	C	N	N	N	N	N	N
4	S	S	N	N	S	N	N	N	N	N	N	N
9	S	S	N	N	N	S	N	N	N	N	N	N
12	N	S	N	N	N	M	C	N	N	N	N	N
20	N	N	N	N	S	N	N	N	N	N	N	N

* Texted three days after drying; kept at room temperature.

† N = no hemolysis; S = slight hemolysis; M = marked hemolysis; C = complete hemolysis.

The results of a similar experiment with sheep cells are shown in table 7; marked deterioration of the natural antisheep hemolysins also occurred but owing to the presence of relatively large amounts of antisheep hemolysin in human sera in addition to a greater resistance of these hemolysins to deterioration (4), many of the dried sera were hemolytic and remained so over a period of at least three weeks.

Additional experiments were conducted by exposing washed sheep corpuscles to equal amounts of the same serum before and after drying at a low temperature followed by removal of the cells and two washings with saline solution and titration of each suspension for the degree of sensitization by distributing the sus-

pensions in doses varying from 0.1 to 1 cc. in test tubes and furnishing complement by adding 1 cc. of a 1:20 dilution of hemolysin free guinea-pig serum. After one hour in a water bath at 38°C. the "hemolytic index" of each serum was read off, that is,

TABLE 7

The influence of drying human serum in paper upon natural antisheep hemolysin

SERA	RESULTS WITH SERA BEFORE DRYING						RESULTS WITH SERA AFTER DRYING*					
	Corpuscles 1	Corpuscles 2	Corpuscles 3	Corpuscles 4	Corpuscles 5	Corpuscles 6	Corpuscles 1	Corpuscles 2	Corpuscles 3	Corpuscles 4	Corpuscles 5	Corpuscles 6
2	C†	C	C	C	C	C	N	N	N	N	N	N
4	M	M	C	M	C	M	N	N	N	N	N	N
5	C	C	C	C	C	C	N	N	S	N	N	N
7	M	M	C	S	C	S	N	N	N	N	N	N
9	C	C	C	C	C	C	N	N	S	N	N	N
10	C	C	C	C	C	C	N	M	M	M	C	M
12	C	C	C	C	C	C	N	N	S	N	M	N
13	C	C	C	C	C	C	N	N	N	N	N	N
17	C	C	C	C	C	C	N	S	S	S	N	N
18	C	C	C	C	C	C	M	M	C	S	C	M

* Tested two days after drying; kept at room temperature.

† C = complete hemolysis; M = marked hemolysis; S = slight hemolysis; N = no hemolysis.

TABLE 8

The influence of drying human serum in paper upon natural antisheep hemolysin

SERA	HEMOLYTIC INDICES OF SHEEP CELLS	
	Sensitized in serum before drying	Sensitized in serum after drying
	cc.	*cc.*
1	0.1	0.5
2	0.1	0.5
3	0.05	None
4	0.1	None

the largest amount of corpuscle suspension showing complete hemolysis, and the method permitted an accurate measure of the degree of deterioration of the hemolysins consequent to drying as measured by the degree of sensitization of the same amounts of corpuscles in the fluid and dried portions of each serum.

The results of experiments of this kind with natural antisheep hemolysins are shown in tables 8 and 9 and with antiguinea-pig hemolysin in table 10. Invariably deterioration of these hemolysins took place as a result of drying which sometimes progressed to a slight extent over a period of three weeks.

TABLE 9

The influence of drying human serum in paper upon natural antisheep hemolysin

SERA	HEMOLYTIC INDICES		
	Before drying	Dried 1 day	Dried 2 days
	cc.	*cc.*	*cc.*
1	0.6	0.4	0.3
2	0.2	None	None
3	0.5	0.1	0.1
4	0.4	0.1	0.1
5	0.3	0.5	None
6	0.4	0.1	None
7	0.3	0.1	None

TABLE 10

The influence of drying human serum in paper upon natural antiguinea-pig hemolysin

SERA	HEMOLYTIC INDICES		
	Before drying	Dried 1 day	Dried 2 days
	cc.	*cc.*	
1	0.2	0.1	None
2	0.1	None	None
3	0.1	0.5	None

CONCLUSIONS

1. Drying normal human sera upon cover glasses and in paper at ordinary room temperatures frequently results in marked or complete deterioration of the normal isohemagglutinins.

2. Deterioration of these normal isohemagglutinins is especially evident within the first to fourth days after the sera have been dried.

3. Similar results were observed with hemagglutinins in normal human sera for the corpuscles of the lower animals.

4. Human sera containing large amounts of normal hemagglutinins when dried under ordinary conditions and properly kept in a refrigerator may prove satisfactory for microscopical tests for at least two weeks, due to the presence of sufficient agglutinins escaping destruction. Only such sera should be used for drying and tests should be made at the end of the first week to determine if agglutinins are present before the cover glasses are used for the typing of bloods.

5. The hemolysins found in normal human sera for the corpuscles of persons and the lower animals also deteriorate upon desiccation under ordinary conditions and are somewhat more susceptible than the hemagglutinins.

6. For the grouping of blood, sera should be kept in a fluid state sealed in ampoules at a low temperature, both hemagglutinins and hemolysins in normal human sera being highly susceptible to heat.

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THE NATURE OF THERMOLABILE HEMOLYSINS[1]

JOHN A. KOLMER

From the Dermatological Research Laboratories of Philadelphia

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Studies bearing upon the presence of natural hemolysins in human serum for the erythrocytes of the lower animals have been largely conducted with heated sera and principally for antisheep hemolysin, owing to the widespread use of the sheep hemolytic system in complement fixation tests; for this reason the natural hemolysins are commonly regarded as thermostabile or heat resistant, as is generally true of the immune hemolysins.

Thiele and Embleton (1) have shown that a portion of the natural antisheep hemolysins present in active human sera and of the immune hemolysins in rabbit sera are thermolabile or heat sensitive, being destroyed or inactivated by heating serum at 56°C. for thirty minutes. These investigators have sought to prove that natural hemolysins and the first production of immune hemolysins are thermolabile and of the nature of differentiated complements, which may be absorbed by the corresponding corpuscles at a low temperature; later in the course of immunization, the immune hemolysins become more and more differentiated from complement becoming thermostabile in nature and removable by absorption with corpuscles without absorption of complement.

Sherman (2), however, claims that all hemolysins, both natural and immune, are thermostabile and that the reduction in hemolytic activity of a serum as a result of heating is due to "masking" of the hemolysin rather than an actual destruction

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This investigation has been conducted through the use of funds accruing from the distribution of arsphenamine.

or inactivation. Sherman designates as "obvious" hemolysis all that are active in heated serum and as "masked" hemolysins, those proving active in unheated but not in heated serum, but the presence of which may be shown by absorption tests, the corpuscles becoming sensitized in heated serum to the same degree as in unheated or active serum. In other words, according to this view, certain hemolysins may become "masked" as a result of heating the sera and incapable of sensitizing erythrocytes in the presence of complement and producing hemolysins during the usual period of incubation of an hour at 38°C. but are

TABLE 1

Summary showing the percentage of human sera in amount of 0.1 cc. containing hemolysins for the erythrocytes of various animals

ERYTHROCYTES	HEMOLYSINS	
	Thermolabile	Thermostabile
	per cent	*per cent*
Human	4 to 44	0 to 4
Sheep	85 to 95	80 to 95
Ox	72 to 88	5 to 30
Guinea-pig	90 to 100	20 to 25
Rabbit	94 to 100	0 to 2
Rat	4 to 32	0 to 2
Hog	92 to 100	0 to 15
Dog	About 98	About 4
Chicken	About 62	0

not actually destroyed being capable of sensitizing corpuscles in mixtures of heated serum and cells, the latter undergoing hemolysis when removed from the serum and mixed with complement.

In a study of the natural hemolysins and hemagglutinins in active and heated human sera in relation to the Wassermann test (3) we have found that a large percentage of active or unheated human sera contain a wide variety of hemolysins for the corpuscles of the lower animals and after heating the sera at 56°C. for thirty minutes the majority of these hemolysins are destroyed or rendered inactive; indeed, the natural hemolysis in human sera for human corpuscles and for those of the guinea-pig, rabbit, rat, dog, hog and chicken are very largely of this

thermolabile variety as shown in a summary given in table 1. This study also showed that the hemolysins and hemagglutinins in human sera for the erythrocytes of the lower animals occur in groups similar to the four groups of isohemagglutinins and that figures based upon work conducted with the corpuscles of any one animal of a species are only approximately correct. The percentages shown in table 1 were secured by testing each serum with corpuscles from at least six different animals of each species and showed the marked variation in the hemolysin content of sera for the corpuscles of any given species.

PURPOSES OF INVESTIGATION

The purposes of this investigation were mainly twofold, namely, to study the relation of the natural hemolysins in human serum to human complement and of antisheep immune hemolysins in rabbit serum to rabbit complement, to determine whether these hemolysins are differentiated complements according to the theory of Thiele and Embleton; secondly, to study the fate of the natural hemolysins in human serum as a result of heating.

There can be no doubt that heating human serum at 56°C. for thirty minutes either destroys outright or inactivates certain hemolysins, notably that for guinea-pig cells; it is commonly believed that they are actually destroyed and I have sought to determine whether this occurs or whether the hemolysins become masked as described by Sherman, without undergoing complete destruction.

PART ONE

Thermolabile and thermostabile hemolysins

In differentiating between the natural thermolabile and thermostabile hemolysins in human sera shown in table 1, all sera were tested fresh and active and again after being heated in a water bath at 56°C. for thirty minutes; with active sera the complement of each serum was utilized while with heated sera complement was furnished by the mixed sera of guinea-pig previously absorbed at a low temperature with the corpuscles in order to remove any traces of hemolysin and hemagglutinin.

This degree of heat is purely arbitrary; deterioration of natural hemolysins is not marked until a serum has been exposed to a temperature of 52° to 56°C. for fifteen to thirty minutes (tables 2 and 3), but at 62°C. deterioration occurs quite rapidly and is generally completed after thirty to forty-five minutes (table 4). In my experience natural antisheep hemolysin is more resistant to heat than any of those studied (tables 3 and 4) and anti-

TABLE 2

The effect of heating human sera at 56°C. upon natural antiguinea-pig hemolysin

SERA	ACTIVE			5 MINUTES			15 MINUTES			20 MINUTES			30 MINUTES		
	0.01	0.1	0.2	0.01	0.1	0.2	0.01	0.1	0.2	0.01	0.1	0.2	0.01	0.1	0.2
1	N*	C	C	N	S	M	N	S	S	N	N	S	N	N	N
2	N	C	C	N	M	C	N	N	S	N	N	N	N	N	N
3	N	C	C	N	M	C	N	N	S	N	N	N	N	N	N
4	N	S	C	N	N	S	N	N	N	N	N	N	N	N	N
5	N	S	C	N	N	N	N	N	N	N	N	N	N	N	N

* C = complete hemolysis; M = marked hemolysis; S = slight hemolysis; N = No hemolysis.

TABLE 3

The effect of heating human sera at 56°C. upon natural antisheep hemolysin

SERA	ACTIVE			5 MINUTES			15 MINUTES			30 MINUTES			60 MINUTES		
	0.01	0.1	0.2	0.01	0.1	0.2	0.01	0.1	0.2	0.01	0.1	0.2	0.01	0.1	0.2
1	S*	C	C	N	S	C	N	S	C	N	S	S	N	S	S
2	C	C	C	C	C	C	C	C	C	S	C	C	S	C	C
3	S	C	C	S	C	C	S	M	C	S	M	M	S	M	M
4	M	C	C	S	C	C	S	M	C	S	S	C	S	S	M

* C = complete hemolysis; M = marked hemolysis; S = slight hemolysis; N = no hemolysis.

guinea-pig hemolysin one of the most susceptible (table 2), but all natural hemolysins are much more susceptible to heat than immune hemolysins and in this regard bear a resemblance to the thermolabile nature of complement. If a temperature of 62°C. and an exposure of one hour were adopted for the differentiation of thermolabile and thermostabile hemolysins, all natural hemolysins in human sera would be classed as thermolabile, but 56°C.

for thirty minutes has been generally adopted and with this exposure all natural hemolysins may be divided into the two classes.

The experiments shown in tables 2, 3 and 4 were conducted with fresh sera; heating was conducted in a water bath. Washed guinea-pig and sheep corpuscles were employed in dose of 1 cc. of 1 per cent suspension with the graded amounts of each serum. Complement was furnished by the mixed hemolysin-free sera of guinea-pigs in dose of 0.2 cc. of 1:10 dilution; the results were read after water bath incubation for one hour.

TABLE 4

The effect of heating human sera at 62°C. upon natural antisheep hemolysin

SERA	ACTIVE			5 MINUTES			15 MINUTES			30 MINUTES			45 MINUTES		
	0.01	0.1	0.2	0.01	0.1	0.2	0.01	0.1	0.2	0.01	0.1	0.2	0.01	0.1	0.2
1	S*	C	C	M	C	C	N	S	M	N	S	S	N	N	N
2	C	C	C	S	M	C	N	S	S	N	N	S	N	N	N
3	S	C	C	S	M	C	N	S	M	N	N	N	N	N	N
4	M	C	C	S	S	M	N	N	S	N	N	S	N	N	N

*C = complete hemolysis; M = marked hemolysis; S = slight hemolysis; N = no hemolysis.

The relative susceptibility to heat of natural hemolysins and complements in human sera

Bearing directly upon the question of the relation of natural hemolysins to complement raised by the work of Thiele and Embleton, is the relative susceptibility of both to heat. As previously stated most of the natural hemolysins are quite susceptible and the thermolability of complement is well known; for these reasons experiments designed to bring out differences in resistance if they exist, required frequent repetition and the closest attention to technical details.

Inasmuch as natural antiguinea-pig hemolysin appears to be very thermolabile, most of my experiments were conducted with pig cells and attempts made to study the comparative resistance of this particular hemolysin and the complement of human sera.

These experiments were conducted by two methods; in the first method fresh human sera were titrated in varying amounts with 1 cc. of a 1 per cent suspension of washed pig cells to determine the smallest amount of serum proving completely hemolytic and also titrated with antihuman hemolysin and 0.1 cc. of a 5 per cent suspension of washed human corpuscles for the unit of complement. The sera were then heated at 56°C. and the titrations were repeated at intervals to determine the relative rates of deterioration of the hemolysin and complement. The results observed with one serum are shown in table 5 and they indicate that the resistance of natural antiguinea-pig hemolysin to heat is but slightly greater than the complement of human serum.

TABLE 5

The influence of heat upon the complement and natural antiguinea-pig hemolysin of human serum

SERUM	HEMOLYTIC ACTIVITY FOR PIG CELLS*				COMPLEMENT ACTIVITY†					
	0.02	0.05	0.1	0.2	0.02	0.04	0.06	0.08	0.1	0.2
Active—unheated	M‡	C	C	C	N	S	C	C	C	C
Heated for 5 minutes	N	N	S	C	N	N	N	S	M	C
Heated for 10 minutes	N	N	N	S	N	N	N	N	N	N
Heated for 15 minutes	N	N	N	N	N	N	N	N	N	N

* Titrated with 1 cc. of a 1 per cent suspension of washed pig cells.

† Titrated with 0.1 cc. of 5 per cent human cells and antihuman hemolysin.

‡ C = complete hemolysis; M = marked hemolysis; S = slight hemolysis; N = no hemolysis.

Similar experiments with natural antisheep hemolysin have, however, usually shown a much greater resistance of the hemolysin as compared with the complement of each serum.

In the second method the hemolytic activity of fresh sera for guinea-pig cells was determined in titrations employing varying amounts of serum with 1 cc. of 1 per cent suspensions of washed pig corpuscles; the sera were then placed in water at 56°C. and titrated at varying intervals being reactivated by the addition of 0.2 cc. of 1:10 dilutions of guinea-pig serum complement free of isohemolysin for the particular cells being employed. Numerous experiments have shown that human serum complement is very

sensitive to heat and that an exposure of five to ten minutes at a temperature of 56°C. results in marked or complete deterioration.

The results of one experiment are shown in table 6; as a general rule an exposure of five minutes completely removed the hemolytic activity of fresh sera for guinea-pig cells, but that this was due to the destruction of the complement rather than of the hemolysin is indicated by the fact that with reactivation of the serum hemolytic activity was not completely lost until the exposure was prolonged to twenty minutes.

TABLE 6

The influence of heat upon thermolabile antiguinea-pig hemolysin

SERA	TITRATIONS WITH 1 CC. OF 1 PER CENT OF PIG CELLS					
	0.01	0.02	0.03	0.05	0.1	0.2
Fresh active serum	N*	S	M	C	C	C
Serum heated for 5 minutes	N	N	N	N	N	N
Serum heated 5 minutes + complement	N	N	N	S	M	C
Serum heated 10 minutes + complement	N	N	N	S	S	M
Serum heated 15 minutes + complement	N	N	N	N	S	S
Serum heated 20 minutes + complement	N	N	N	N	N	S
Serum heated 30 minutes + complement	N	N	N	N	N	N

* N = no hemolysis; S = slight hemolysis; M = marked hemolysis; C = complete hemolysis.

† Complement furnished as 0.2 cc. of 1:10 pig serum.

Experiments conducted after these methods indicate therefore, that the natural hemolysins are more resistant to heat than the complements in fresh human sera.

The relative susceptibility to age of natural hemolysins and complements in human sera

Experiments conducted in exactly the same manner as described above have shown that the natural antiguinea-pig and antisheep hemolysins in human sera are more resistant to the deterioration of age than the complements of the same sera. In conducting these experiments sterile sera only were employed

in order to prevent the development of anticomplementary substances and the titrations were made at intervals of one, three, five, seven and ten days with sera kept at ordinary room temperature and at 7° to 9°C. in a refrigerator. While the complements had usually completely disappeared within three days in sera kept at room temperature and in five to seven days in sera kept in the refrigerator, the natural hemolysins had undergone only partial deterioration, the sera being reactivated with hemolysin free guinea-pig serum complement as described above.

TABLE 7

The relation of the complement activity of human serum to its hemolytic activity for guinea-pig erythrocytes

SERUM NUMBER	TITRATION OF COMPLEMENT*										TITRATION OF HEMOLYTIC ACTIVITY									
	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.1	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.1
1	N†	N	M	C	C	C	C	C	C	C	N	N	S	M	C	C	C	C	C	C
2	N	N	N	S	C	C	C	C	C	C	N	S	M	C	C	C	C	C	C	C
3	N	N	S	M	M	C	C	C	C	C	N	S	M	C	C	C	C	C	C	C
4	N	N	S	M	C	C	C	C	C	C	N	N	N	S	C	C	C	C	C	C
5	N	N	N	N	S	C	C	C	C	C	N	N	S	C	C	C	C	C	C	C
6	N	N	S	C	C	C	C	C	C	C	N	N	S	C	C	C	C	C	C	C

* With 0.1 cc. of 5 per cent human cells and two units of antihuman hemolysin.

† N = no hemolysis; S = slight hemolysis; M = marked hemolysis; C = complete hemolysis.

The relation of the complement activity of human serum to natural hemolysins

For the purpose of further study of the possible relationship of the natural hemolysins to the complements, fresh human sera were titrated for complement activity in an antihuman hemolytic system and for natural antiguinea-pig hemolysin by using varying amounts of serum with 1 cc. of 1 per cent suspension of guinea-pig cells. The purpose of these experiments was to determine whether sera particularly rich in complement for sensitized human corpuscles were likewise markedly hemolytic for guinea-pig cells or whether sera poor in complement were likewise poor in hemolytic activity for the guinea-pig cells.

The results of experiments with six sera shown in table 7 indicate that the two properties are not absolutely parallel. Sera 3 and 5, for example, were slightly below the average in complement activity while their hemolytic activity for guinea-pig cells was quite marked; serum 1, on the contrary, was quite rich in complement and below the average in hemolysin content. The differences, however, were never marked and were brought out only by strict attention to technical details but I believe the results warrant the conclusion on the basis of the technic employed, that complements and natural hemolysins in human sera are not necessarily present in parallel amounts.

The influence upon the complement sera of the removal by absorption with erythrocytes

Thiele and Embleton have based part of their claim for the existence of thermolabile hemolysins and their relation to complement upon the observation that the addition of complement increases the hemolytic power of an active serum for sheep erythrocytes. This is undoubtedly true but the deductions are not necessarily so; as shown in tables 8 and 9 the addition of hemolysin-free guinea-pig complement to active human and rabbit immune sera increased the hemolytic activity of these sera, but inasmuch as the guinea-pig complements were previously absorbed in the cold with sheep corpuscles and proven free of hemolysin for these cells in doses as large as 0.5 cc., I ascribe the results to the mechanism of the well known phenomenon that an excess of complement will, to a certain extent, increase hemolysis in any hemolytic system although, in view of our ignorance regarding the true nature of serum complement and the mechanism of its activity, the explanation of why this occurs is only a conjecture.

Another of the arguments given by Thiele and Embleton as support for their theory that hemolysins are differentiated complements and that the immune hemolysins are products of evolution from complement, is that absorption of sera at a low temperature with erythrocytes results not only in the removal

of hemolysin but of complement as well and that the washed sensitized corpuscles will undergo some hemolysis in the test tube due to complement and hemolysin they have absorbed. In their experiments it was found that the absorption of normal sera at a low temperature with sheep erythrocytes removed at

TABLE 8

The influence of complement upon the hemolytic activity of human serum

SERUM	FOR 5 PER CENT WASHED SHEEP CELLS										FOR 5 PER CENT WASHED PIG CELLS									
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Active serum 0.1 cc.	C†	C	C	M	S	S	S	S	S	S	C	C	M	M	S	S	S	S	S	S
Active serum 0.1 cc. + complement*	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	M	M	M	M	M
Heated serum 0.1 cc. + complement	C	C	C	C	C	M	S	S	S	S	N	N	N	N	N	N	N	N	N	N

* 0.5 cc. of 1:10 dilution pig serum (hemolysin free).

† C = complete hemolysis; M = marked hemolysis; S = slight hemolysis.

TABLE 9

The influence of complement upon the hemolytic activity of rabbit antisheep serum

SERUM	10 PER CENT SUSPENSION SHEEP CELLS									
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Unheated serum 0.1 cc.	C	C	C	M	M	M	M	S	S	S
Unheated serum 0.1 cc. + complement*	C	C	C	C	C	C	C	C	C	C
Heated serum 0.1 cc. + complement	C	M	M	M	M	S	S	S	S	S

* 0.5 cc. of 1:10 hemolysin free pig serum.

the same time all or a part of the complement, indicating that "the hemolytic substance appears to be complement so modified that it can combine directly with the red cells in the cold and directly cause hemolysis." With the sera of rabbits immunized with human corpuscles, these investigators found that absorption in the cold removed the immune or thermostabile hemolysin

and less and less complement as immunization proceeded or, in other words, that the first production of immune hemolysin in rabbits is thermolabile and allied to the complement, absorption removing not only the hemolysin but a part of all of the complement as well, whereas later, when the immune hemolysin became thermostabile, absorption resulted in the removal of the hemolysin but little or none of complement.

In my experiments fresh human and rabbit immune sera were chilled, treated with chilled washed erythrocytes and the mixtures were maintained just above the freezing point for two

TABLE 10

The influence on the complement of human serum of the removal of natural antisheep hemolysins

SERUM	COMPLEMENT ACTIVITY*										HEMOLYTIC ACTIVITY FOR SHEEP CELLS									
	0..01	0..02	0..03	0..04	0..05	0..06	0..07	0..08	0..09	0.1	0..01	0..02	0..03	0..04	0..05	0..06	0..07	0..08	0..09	0.1
No. 1. Untreated Serum.....	N	S	M	M	C	C	C	C	C	C	N	N	N	S	C	C	C	C	C	C
No. 1. Treated serum.....	N	N	S	M	M	C	C	C	C	C	N	N	N	N	N	N	N	N	N	N
No. 2. Untreated serum.....	N	N	S	M	M	C	C	C	C	C	N	N	S	C	C	C	C	C	C	C
No. 2. Treated serum.....	N	N	S	S	M	M	M	C	C	C	N	N	N	N	N	N	N	N	N	N

* Titrated with 0.1 cc. of 5 per cent washed sheep cells and antihuman hemolysin.

hours followed by rapid separation of the cells and serum in centrifuge cups packed with mixtures of ice-snow; this low temperature was maintained throughout to prevent hemolysis but the operation was not always successful, many of the experiments requiring frequent repetition for correct technical conditions. Each serum was titrated before and after treatment with corpuscles for complement by using varying amounts of serum with a fixed amount of inactivated rabbit antihuman hemolysin and 0.1 cc. of 5 per cent human corpuscle suspension, to determine whether complement was absorbed along with the hemolysins.

In table 10 are shown the results of experiments with two fresh human sera from which all of the natural antisheep hemolysin

had been removed; in each case the complement activity was slightly diminished after absorption of the sera with sheep cells.

In table 11 are shown the results of experiments with three fresh human sera from which most or all of the natural anti-guinea-pig hemolysin had been removed;[2] in these tests the complements of the treated sera were not diminished.

In other experiments the complement activity of fresh normal human and fresh immune rabbit sera was determined in the same manner in an antihuman hemolytic system before and after treatment with washed corpuscles, while the tests for hemolysin were conducted by using each serum in a constant

TABLE 11

Influence of the removal of natural antiguinea-pig hemolysin from active human sera upon complement

SERA	COMPLEMENT ACTIVITY*.		HEMOLYTIC ACTIVITY†	
	Unit before absorption	Unit after absorption	Unit before absorption	Unit after absorption
	cc.	*cc.*	*cc.*	*cc.*
1	0.04	0.04	0.05	More than 0.1
2	0.05	0.04	0.04	More than 0.1
3	0.06	0.06	0.04	0.1

* Titrated with 0.1 cc. of 5 per cent suspension human corpuscles and two units of antihuman hemolysin.

† Smallest amounts of serum producing complete hemolysis of 0.1 cc. of 5 per cent guinea-pig cells.

dose of 0.1 cc. with increasing amounts of 5 per cent suspensions of corpuscles; the results of experiments with human sera from which the natural antisheep and antiguinea-pig hemolysins were removed are shown in table 12 indicating that the corpuscles had removed none or but traces of complement whereas all of the hemolysins were absorbed. Table 13 shows the results of similar experiments with the sera of rabbits after the intravenous injection of one, two and three doses of washed sheep cells in amounts of 5 cc. of 10 per cent suspensions at intervals of five

[2] In this connection, I may state that the removal of antiguinea-pig hemolysin from human sera by absorption with pig erythrocytes in the cold, usually requires a longer period than does the removal of antisheep hemolysin by sheep corpuscles.

TABLE 12

Influence of the removal of natural hemolysins from active human sera upon complement

SERA	HEMOLYSIN	COMPLEMENT ACTIVITY*		HEMOLYTIC ACTIVITY†	
		Unit of serum before absorption	Unit of serum after absorption	Unit of cells before absorption	Unit of cells after absorption
		cc.	cc.	cc.	
1	Antiguinea-pig	0.04	0.06	0.2	None
1	Antisheep			0.3	None
2	Antiguinea-pig	0.03	0.03	0.3	None
2	Antisheep			0.2	None
3	Antiguinea-pig	0.05	0.05	0.3	None
3	Antisheep			0.3	None
4	Antiguinea-pig	0.05	0.05	0.3	None
4	Antisheep			0.3	None

* Titrated with 0.1 cc. of 5 per cent suspension of human corpuscles and two units of antihuman hemolysin.

† Largest amounts of 5 per cent suspensions of sheep and guinea-pig cells completely hemolysed by 0.1 cc. of serum before and after absorption.

TABLE 13

Influence of the removal of immune antisheep hemolysin from active rabbit serum upon complement

SERA	NUMBER OF INJECTIONS OF SHEEP CELLS	COMPLEMENT ACTIVITY*		HEMOLYTIC ACTIVITY†	
		Unit of serum before absorption	Unit of serum after absorption	Unit of cells before absorption	Unit of cells after absorption
		cc.	cc.	cc.	
1	1	0.05	0.06	0.2	None
2	1	0.05	0.05	0.2	None
1	2	0.05	0.08	0.4	None
2	2	0.05	0.1	0.4	None
1	3	0.04	0.06	0.7	None
2	3	0.05	0.08	0.7	None

* Titrated with 0.1 cc. of 5 per cent suspension of human corpuscles and two units of antihuman hemolysin.

† Largest amounts of a 10 per cent suspension of sheep corpuscles completely hemolysed by 0.1 cc. rabbit immune serum.

days; in these experiments there were in some instances an apparent drop in the amount of complement in the sera after absorption of the hemolysins, but it was exceedingly difficult to prevent hemolysis during the process of absorption and I believe the slight loss of complements in the treated sera can be surely ascribed to their utilization in the production of this slight degree of hemolysis.

TABLE 14

Concerning the absorption of complement and hemolysins by erythrocytes from active sera

SERA	SERA ABSORBED BY	BEHAVIOR OF WASHED CORPUSCLES	
		Corpuscles alone	Corpuscles with complement*
Rabbit anti-sheep, 1 injection..	Sheep cells	No hemolysis	Complete hemolysis
Rabbit anti-sheep, 1 injection..	Sheep cells	No hemolysis	Complete hemolysis
Rabbit anti-sheep, 2 injections.	Sheep cells	No hemolysis	Complete hemolysis
Rabbit anti-sheep, 2 injections.	Sheep cells	No hemolysis	Complete hemolysis
Rabbit anti-sheep, 3 injections.	Sheep cells	No hemolysis	Complete hemolysis
Rabbit anti-sheep, 3 injections.	Sheep cells	No hemolysis	Complete hemolysis
Normal human.....	Sheep cells	No hemolysis	Partial hemolysis
Normal human.....	Sheep cells	No hemolysis	Partial hemolysis
Normal human.....	Sheep cells	No hemolysis	Partial hemolysis
Normal human.....	Sheep cells	No hemolysis	Partial hemolysis
Normal human.....	Guinea-pig cells	No hemolysis	Partial hemolysis
Normal human.....	Guinea-pig cells	No hemolysis	Partial hemolysis
Normal human.....	Guinea-pig cells	No hemolysis	Partial hemolysis
Normal human.....	Guinea-pig cells	No hemolysis	Partial hemolysis

* Furnished by 1 cc. of 1:20 hemolysin-free guinea-pig complement.

According to my experiments therefore, complement is not absorbed by corpuscles; where a serum lost slightly in hemolytic activity after being treated with corpuscles, the loss was due to the consumption of a portion of the complement in the hemolysis of sensitized cells, this being very difficult to check and especially in the presence of large amounts of natural or immune antisheep hemolysin in human and rabbit sera.

In all experiments the sheep and guinea-pig corpuscles used for the absorption of the respective hemolysins were washed twice with iced physiological salt solution and then resuspended in warm salt solution; in no instance were these corpuscles ob-

served to undergo hemolysis following incubation in a water-bath at 38°C. for one hour, as found by Thiele and Embleton. In other words, I could find no evidence that these cells had bound complement, although they were well sensitized as shown by the occurrence of partial or complete hemolysis upon the addition of hemolysin free guinea-pig serum complement (table 14).

The influence of filtration of active human sera upon complement and natural hemolysins

More conclusive evidence that the natural hemolysins of human sera are not related to the complements, was found in a series of filtration experiments; filtering fresh human sera

TABLE 15

The influence of filtration upon complement and antisheep hemolysin in human serum

TEST	RESULTS OF TITRATION WITH 0.1 CC. OF 5 PER CENT SHEEP CELLS									
	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.1
Unfiltered serum	N±	S	S	M	M	C	C	C	C	C
Filtered serum	N	N	N	N	N	N	N	N	N	N
Filtered + complement*	N	S	S	M	M	M	C	C	C	C
Filtered serum + hemolysin†	N	N	N	N	N	N	N	N	N	N

* 0.2 cc. of 1:10 guinea-pig serum.

† Two units of antisheep hemolysin.

‡ N = no hemolysis; S = slight hemolysis; M = marked hemolysis; C = complete hemolysis.

through new, sterile and chemically clean Kitasato earthen filters removed all complement but none or only slight amounts of the natural antisheep and antiguinea-pig hemolysins.

The results observed with one of several sera are shown in table 15; 0.06 cc. of this serum produced complete hemolysis of 0.1 cc. of a 5 per cent suspension of sheep cells. After filtering 2 cc. of this serum its hemolytic activity was lost due to the removal of complement by the filter but none or only a trace of the hemolysin, inasmuch as the addition of complement restored hemolytic activity whereas the addition of hemolysin did not (table 15).

The removal of a large portion or all of the complement from human sera by these filters is further shown in table 16; the sera were titrated in varying amounts with inactivated antihuman hemolysin and 0.1 cc. of a 5 per cent suspension of human corpuscles before and after filtration, the first one or at most two cubic centimeters passing the filter being employed.

TABLE 16

The influence of filtration upon complement

SERA	BEFORE FILTRATION						AFTER FILTRATION					
	0.01	0.03	0.04	0.06	0.08	0.1	0.01	0.02	0.04	0.06	0.08	0.1
1	M*	C	C	C	C	C	N	N	N	N	N	N
2	M	M	C	C	C	C	N	N	S	S	S	M
3	S	M	C	C	C	C	N	N	N	N	S	S
4	S	M	C	C	C	C	N	N	N	N	N	S

* C = complete hemolysis; M = marked hemolysis; S = slight hemolysis; N = no hemolysis.

TABLE 17

The influence of filtration upon complement and antiguinea-pig hemolysin in human serum

TEST	RESULTS OF TITRATION WITH 1.0 CC. OF 1 PER CENT PIG CELLS									
	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.1
Unfiltered serum	N†	N	S	S	M	M	C	C	C	C
Filtered serum	N	N	N	N	N	N	N	N	N	N
Filtered serum + complement*	S	M	C	C	C	C	C	C	C	C

* 0.1 cc. of 1:20 guinea-pig serum.

† N = no hemolysis; S = slight hemolysis; M = marked hemolysis; C = complete hemolysis.

The results of an experiment in which fresh human serum and guinea-pig corpuscles were employed is shown in table 17; this serum proved completely lytic for 1 cc. of a 1 per cent suspension of guinea-pig corpuscles in dose of 0.07 cc. After filtration its hemolytic activity was lost due to the removal of the complement by the filter; the addition of a small dose of guinea-pig complement previously absorbed with the same corpuscles to remove any isohemolysin which may have been present, restored hemolytic activity beyond that of the unfiltered serum.

PART TWO

The absorption of natural hemolysins from active and heated human sera

Numerous experiments have been conducted to determine whether certain natural hemolysins in fresh human sera are actually destroyed by heating or simple inactivated or masked as described by Sherman; these experiments were conducted as follows with each serum after preliminary tests had shown the presence of hemolysin:

1. With each fresh unheated serum 0.1 cc. was placed in a series of ten test tubes; increasing amounts of 5 per cent suspension of washed corpuscles varying from 0.1 to 1 cc. were added and the volume of each tube was equalized with salt solution. After water bath incubation for one hour the results were read and the *largest dose of corpuscles completely hemolysed was designated as the hemolytic index of the serum*, hemolysis being due to the activity of natural hemolysin and native complement.

These tests were then repeated with sera heated at 56° and 62°C. for thirty minutes in exactly the same manner except that hemolysin-free complement was furnished in dose of 1 cc. of 1:20 dilution of guinea-pig serum absorbed by the corpuscles at a low temperature over night; the results showed the degree of inactivation or destruction of hemolysin as the result of heating.

2. One cubic centimeter of each active or unheated serum was now chilled and treated with 4 cc. of 5 per cent suspension of cells (also chilled) and the mixture kept at about the freezing point over night; the next morning the cells were removed by centrifuging and washed twice with iced salt solution. After the last washing the cells were suspended in 4 cc. salt solution and distributed in doses ranging from 0.1 to 1 cc. in a series of ten test tubes; complement previously absorbed with corpuscles for the natural hemolysin under study, was added in constant dose of 1 cc. of 1:20 dilution. After one hour in a water bath the results were read and the *largest dose of corpuscles showing complete hemolysis was designated as the hemolytic index of cor-*

puscles and giving an exact measure of the degree of sensitization. These tests were repeated in exactly the same manner with portions of each serum heated at 56° and 62°C. for thirty minutes. With this technic it was possible to study the influence of heat upon natural hemolysins as determined in the usual

TABLE 18

The absorption of natural antisheep hemolysin from active and heated serum

SERUM NUMBER	HEMOLYTIC ACTIVITY OF 5 PER CENT SUSPENSIONS OF CORPUSCLES AFTER EXPOSURE TO ACTIVE SERA										HEMOLYTIC ACTIVITY OF 5 PER CENT SUSPENSIONS OF CORPUSCLES AFTER EXPOSURE TO HEATED SERA									
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
1	C*	C	C	C	M	M	M	M	M	M	M	S	S	S	S	S	S	S	S	S
2	C	M	S	S	S	S	S	S	S	S	N	N	N	N	N	N	N	S	S	S
3	C	C	C	C	C	M	M	M	M	M	C	C	M	M	M	S	S	S	S	S
4	M	M	S	S	S	S	S	S	S	S	N	N	N	N	N	N	N	N	N	S
5	C	C	M	M	M	M	M	M	M	M	M	M	M	M	S	S	S	S	S	S
6	C	C	C	M	M	S	S	S	S	S	C	M	S	S	S	S	S	S	S	S
7	C	C	C	C	M	M	M	M	M	M	M	S	S	S	S	S	S	S	S	S
8	C	C	M	M	M	M	M	M	M	M	S	S	S	S	S	S	S	S	S	S

*C = complete hemolysis.

TABLE 19

The absorption of natural antisheep hemolysin from unheated and heated human sera

SERA	HEMOLYTIC INDICES OF CORPUSCLES AFTER ABSORPTION			HEMOLYTIC INDICES OF SERA		
	Unheated	56°C.*	62°C.*	Unheated	56°C.*	62°C.*
1	0.5	0.5	0	0.4	0.2	0
2	0.5	0.5	0.2	0.5	0.3	0.1
3	0.5	0.5	0.2	0.5	0.2	0.1
4	0.5	0.5	0	0.4	0.2	0
5	0.5	0.5	0	0.5	0.1	0
6	0.2	0	0	0.3	0	0

* Sera heated at 56°C. and 62°C. for thirty minutes.

manner by direct titration and also by determining the degree of sensitization occurring in unheated and heated sera under identical conditions.

These experiments were conducted with ten to twenty-five human sera for natural antisheep, antiox, antiguinea-pig, anti-

rat, antidog and antirabbit hemolysins and the results observed with three to nine sera are shown in tables 18 to 24 as examples of each series; these results may be summarized as follows:

1. A general statement on the fate of natural hemolysins as a result of heating the sera cannot be made, as the various hemolysins behave somewhat differently by reason of variation in resistance to heat.

2. Antisheep hemolysin is most resistant to heat; after an exposure of serum to 56°C. for thirty minutes the hemolytic index is reduced although corpuscles are usually sensitized to the same degree as in unheated serum. After exposure to 62°C.

TABLE 20

The absorption of natural antiox hemolysin from unheated and heated human sera

SERA	HEMOLYTIC INDICES OE CORPUSCLES AFTER ABSORPTION			HEMOLYTIC INDICES OF SERA		
	Unheated	50°C.	62°C.	Unheated	56°C.*	62°C.*
1	0.1	0.1	0	0.1	0	0
2	0.2	0.1	0	0.2	0.1	0
3	0.5	0.3	0	0.5	0.2	0
4	0.4	0.2	0	0.3	0.1	0
5	0.1	0.1	0	0.1	0	0
6	0.3	0.1	0	0.2	0.1	0

* Sera heated at 56°C. and 62°C. for thirty minutes.

for thirty minutes the hemolytic activity is lost or greatly reduced and absorption removes a proportionately smaller amount of hemolysin or none at all (tables 18 and 19).

3. Antiox hemolysin behaves in a similar manner; the hemolytic indices of sera heated at 56°C. for thirty minutes are lower than unheated sera and totally lost after heating at 62°C. Absorption of sera heated at 56°C. shows the same or slightly less sensitization than occurs in unheated serum; absorption of sera heated at 62°C. showed no sensitization (table 20).

4. Antiguinea-pig hemolysin is usually destroyed by heating sera at 56°C. for thirty minutes and always after heating at 62°C. for the same period. Corpuscles are sensitized very slowly in unheated sera at low temperatures and agglutination

frequently occurs; under identical conditions, I have only occasionally found corpuscles sensitized in sera heated at 56°C. and never after heating at 62°C. (table 21).

5. With antirat, antidog and antirabbit hemolysins heating sera at 56°C. for thirty minutes generally reduced or totally removed hemolytic activity although the corpuscles were sensitized to the same or slightly less degree than occurred in unheated sera; after heating at 62°C. for thirty minutes hemolytic activity of the sera was totally destroyed and the sera failed to sensitize corpuscles (tables 22, 23 and 24).

TABLE 21

The absorption of natural antiguinea-pig hemolysin from unheated and heated human sera

SERA	HEMOLYTIC INDICES OF CORPUSCLES AFTER ABSORPTION			HEMOLYTIC INDICES OF SERA		
	Unheated	56°C.	62°C.	Unheated	56°C.	62°C.
1	0.1	0	0	0.1	0	0
2	0.1	0	0	0.2	0	0
3	0.1	0	0	0.3	0	0
4	0.2	0.1	0	0.3	0.1	0
5	0	0	0	0.1	0	0
6	0.1	0	0	0.3	0	0
7	0.1	0	0	0.1	0	0
8	0.2	0	0	0.3	0.1	0
9	0.2	0	0	0.3	0	0

The general results of these experiments were to show that apparently a portion of the natural hemolysin in human sera for the corpuscles of the sheep, ox, guinea-pig, rat, dog and rabbit may become masked or inactivated as described by Sherman, that is, while the hemolytic activity of the serum is reduced in a direct titration, the corpuscles become sensitized to the same degree as in unheated serum; but a portion of some hemolysins are actually destroyed by heating at 56°C. for thirty minutes as shown by the fact that sensitization is less or nil in heated serum as compared with unheated serum and this is particularly true of such hemolysins as those for guinea-pig cells which are more sensitive to heat than those for sheep and ox corpuscles.

As is well known, sensitized corpuscles are more vulnerable to complement and hemolysis than plain cells mixed with the same amount of hemolysin and complement and "masking" of hemolysins by heat may be due to the production of amboceptoids

TABLE 22

The absorption of natural antirat hemolysin from unheated and heated human sera

SERA	HEMOLYTIC INDICES OF CORPUSCLES AFTER ABSORPTION			HEMOLYTIC INDICES OF SERA		
	Unheated	56°C.	62°C.	Unheated.	56°C.	62°C.
1	0.1	0.1	0	0.1	0	0
2	0.1	0.1	0	0.1	0.1	0
3	0.1	0.1	0	0.1	0	0

TABLE 23

The absorption of natural antidog hemolysis from unheated and heated human sera

SERA	HEMOLYTIC INDICES OF CORPUSCLES AFTER ABSORPTION			HEMOLYTIC INDICES OF SERA		
	Unheated	56°C.	62°C.	Unheated	56°C.	62°C.
1	0.2	0.2	0	0.2	0.1	0
2	0.1	Trace	0	0.2	0	0
3	Trace	Trace	0	0.1	0	0

TABLE 24

The absorption of natural antirabbit hemolysin from unheated and heated human sera

SERA	HEMOLYTIC INDICES OF CORPUSCLES AFTER ABSORPTION			HEMOLYTIC INDICES OF SERA		
	Unheated	56°C.	62°C.	Unheated	56°C.	62°C.
1	0.1	0	0	0.2	0	0
2	0.2	0.1	0	0.3	0.1	0
3	0.1	0	0	0.2	0.1	0

which block unchanged hemolysins in the short exposure of one hour whereas in prolonged absorption of heated sera the cells may become sensitized with sufficient unchanged amboceptor to render them as vulnerable to complement as cells exposed to unheated serum; according to my experiments heating sera at 56 and 62°C. results in an actual destruction as well as inactiva-

tion or masking of natural hemolysins, the different hemolysins found in human sera varying considerable in resistance to these changes.

SUMMARY

1. Natural hemolysins in human sera are more resistant to heat and age than complement.

2. There is no relation between the complement content and natural hemolysins in human sera.

3. Absorption of active human sera with corpuscles removes hemolysin but not complement.

4. Washed sensitized cells have not absorbed complement and do not undergo hemolysis unless complement is furnished.

5. Filtration may remove complement from a serum without any or but slight removal of a natural hemolysin.

6. Heating serum sera at 56°C. for thirty minutes results in the partial destruction and inactivation ("masking") of natural hemolysins, the different hemolysins varying in their resistance; heating at 62°C. results in the destruction of natural hemolysins.

CONCLUSIONS

1. These experiments indicate that the natural hemolysins in human sera are distinct substances and not differentiated complements.

2. Natural hemolysins are susceptible to heat being inactivated (masked) or destroyed when sera are heated at 56°C. and totally destroyed by heating at 62°C. The natural hemolysins in human sera vary in resistance to heat, antisheep hemolysin being most resistant (thermostabile) and antiguinea-pig hemolysin being most susceptible (thermolabile).

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COMPLEMENTARY AND OPSONIC FUNCTIONS IN THEIR RELATION TO IMMUNITY

A STUDY OF THE SERUM OF GUINEA-PIGS NATURALLY DEFICIENT IN COMPLEMENT

HIRAM D. MOORE

From the Veterinary Laboratory of the Vermont State Agricultural Experiment Station, Burlington, Vermont

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SOME TITRATIONS OF GUINEA-PIG COMPLEMENT

Several years ago, it was discovered in the Veterinary Department of the Vermont State Agricultural Experiment Station, in connection with the work on the complement fixation test, that the blood serum of some guinea-pigs is very deficient in complement. The late Ramon C. Downing found that in some instances, as much as 1 cc. of guinea-pig serum, which he tested, produced no hemolysis of sensitized corpuscles. In the course of his work, Downing used two different hemolytic systems, one with sheep erythrocytes, the other with horse erythrocytes. His work is unpublished.

At the time that this deficiency of complement in the serum of some guinea-pigs was discovered, Dr. F. A. Rich, head of the Veterinary Department of the Vermont State Agricultural Experiment Station, and Ramon C. Downing began a breeding experiment with such animals. The results of this experiment have not yet been published; I may say, however, that this deficiency in complement was found to be a heritable condition. As far as I am aware, the complement-deficient guinea-pigs at this Experiment Station are the only ones of the kind in existence; we have been able to multiply them by careful breeding and we have hundreds of them at present.

The writer's tests with the hemolytic systems used by Downing have confirmed this remarkable deficiency in complement. The

sera of several hundred guinea-pigs have been tested, which showed practically no hemolysis with 1 cc. of undiluted serum. Since most of the sera of the complement-deficient guinea-pigs, which we have, show practically no hemolysis in a quantity of 1 cc., we usually made the rough test shown in table 1 in order to determine whether the sera were or were not deficient in complement.

In a few instances the serum of guinea-pigs that had been found deficient in complement when tested with sensitized horse's erythrocytes, were also tested with sensitized human blood corpuscles. Most of the sera so examined were found to be more

TABLE 1

Rough test of the sera of complement deficient guinea-pigs. 0.5 cc. of 1 per cent washed horse erythrocytes and three units of amboceptor in each tube

GUINEA-PIG NUMBER	1.0 cc.	0.3 cc.	0.4 cc.
1	0	0	0
2	+	0	0
3	0	0	0
4	0	0	0
5	0	0	0
6 (normal)	++	++	++

++ = Complete hemolysis; + = partial hemolysis; 0 = no hemolysis.

* Over one thousand guinea-pigs, whose undiluted sera showed practically no hemolysis in a quantity of 1 cc. when titrated in this manner, have been tested by the writer.

deficient in complement for the former system than for the latter. The result of this experiment, the protocol of which is presented in tables 2 and 3, seems to offer further evidence of a multiplicity of complement.

In a further experiment a mixture of the sera of seven guinea-pigs, all of which had been found entirely wanting in complementary power with sensitized horse's corpuscles, was tested with 1 cc. of washed sensitized human corpuscles. With this hemolytic system, also, a deficiency of complementary action was demonstrable; 0.1 cc. was found to be the minimal complementary dose as compared with the normal dose of 0.02 cc.

On the other hand the complementary action of these sera was certainly quantitatively greater toward human cells than it was toward the horse corpuscles, and the difference was possibly a qualitative one. No hemolysis whatever was produced in 1 cc. of 1 per cent sensitized horse corpuscles by 1 cc. of any of the undiluted sera.

TABLE 2

Rough test of the sera of complement deficient guinea-pigs. 0.5 cc. of 1 per cent washed horse erythrocytes and three units of ambocepter in each tube

GUINEA-PIG NUMBER	DEGREE OF HEMOLYSIS CAUSED BY THE UNDILUTED SERA					
	1.0 cc.	0.3 cc.	0.04 cc.	0.02 cc.	0.01 cc.	0.1 cc.*
688	0	0	0	0	0	0
31G	0	0	0	0	0	0
98Z	0	0	0	0	0	0
722	++	0	0	0	0	0
494	++	0	0	0	0	0
716	++	0	0	0	0	0
68C	++	++	0	0	0	0
73G (normal)	++	++	++	++	+	0

* 0.1 cc. of normal serum and 0.5 cc. of 1 per cent washed unsensitized horse erythrocytes in this tube.

TABLE 3

Rough test of the sera of complement deficient guinea-pigs. 1 cc. of 1 per cent washed human erythrocytes and two units of amboceptor in each tube

GUINEA-PIG NUMBER	DEGREE OF HEMOLYSIS CAUSED BY THE UNDILUTED SERA			
	1.0 cc.	0.3 cc.	0.04 cc.	0.02 cc.
688	++	++	0	0
31G	++	++	0	0
98Z	++	++	0	0
722	++	++	0	0
494	++	++	0	0
716	++	+	0	0
68C	++	++	0	0
73G (normal)	++	++	++	++

The question suggested itself whether the complement deficient guinea-pigs differ from the normal animals in their resistance to bacterial infection, and this question was experimentally examined. In one parallel series of experiments, no difference

was found between the complement deficient and the normal guinea-pigs in their ability to produce complement fixing and agglutinative antibodies upon the repeated injection of *killed* bacteria. The appropriate examinations of the sera of the complement deficient animals, after the immunizing injections had been made, showed no increase in the complement content of the blood. This result confirms the original observations of Von Dungern, that the complement content of the blood is not affected by the process of antibody formation.

On the tenth day after the last injection of the killed organism all of the animals of this series were given a small dose; (one-tenth of a twenty-four hour agar slant culture) of the *live* bacteria.

As a result of this inoculation, one complement normal pig died, whereas all of those deficient in complement survived. On April 14 another inoculation was given, this time one-half agar slant culture of living bacilli and three-fifths agar slant culture of living cocci. As a result of this inoculation three complement-deficient animals and four complement-normal animals died. This experiment seemed to show quite conclusively, that the deficiency in complement did not interfere with immunity acquired through a systematic immunization. Subsequent results have confirmed this conclusion.

In a second experiment, a comparative test of the resistance to infection of non-immunized guinea-pigs of the two kinds (complement-deficient and complement-normal) was made. In this test live cultures of *Bacillus cholerae suis* were used. Of 100 complement-deficient guinea-pigs 77 succumbed to the inoculation of the live bacteria, whereas only 20 of the 100 complement-normal guinea-pigs that were similarly inoculated died. This result seems to indicate that with the noted deficiency of complement, there is an associated deficiency in natural resistance to artificial bacterial infection. While it may be thought that this deficiency in natural resistance to bacterial infection is directly due to the deficiency in complement, it is conceivable that other factors that may be concerned in the mechanism of natural immunity, are lacking in these complement-deficient animals, and that the observed deficiency in natural immunity is due to the lack of these other factors.

In view of this possibility and in view of the known presence in normal blood of opsonins, to which have been ascribed some property of resistance to bacterial invasion, the present comparative study of the opsonin content of the blood of the complement-deficient guinea-pigs was undertaken. In this study the complement-deficient sera employed were such as caused no hemolysis of 0.5 cc. of 1 per cent doubly sensitized horse corpuscles in a quantity of 1 cc. of the undiluted serum, whereas the normal sera caused complete hemolysis of the same corpuscular unit in a quantity of 0.06 cc. of a 20 per cent dilution.

The technic of the opsonin determinations followed throughout the study was practically identical with that of Wright with some minor differences.

The bacterial emulsion was made from cultures of the typhoid bacillus.

In the earlier experiments horse leucocytes washed four times in 0.9 per cent saline solution, were used.

For the further determinations leucocytes from the complement-deficient guinea-pigs were used with complement-deficient serum, while leucocytes from normal guinea-pigs were used with complement-normal serum.

The sera to be examined were prepared from blood that had been secured by heart puncture with hypodermic needle; the sera were employed undiluted. They were drawn into a Wright pipet with equal volumes of the washed leucocytes suspension and the bacterial emulsion and then well mixed on a glass slide in the usual manner. The mixture, after being drawn into the pipet, the end of which was then sealed in a flame, was incubated for exactly fifteen minutes at 37°C. At the end of this period the seal was broken and the contents of the pipet was thoroughly mixed again and evenly spread on an absolutely clean glass slide, with the end of another glass slide in the usual manner of spreading a blood film. The smear was air-dried and then stained with Jenner's stain. In the determinations all of the bacteria in 100 leucocytes were counted in every smear. The results of these determinations are presented in table 4.

TABLE 4

Showing the opsonic index of complement-deficient guinea-pigs

GUINEA-PIG		TOTAL	INDEX
1 Normal	6, 6, 9, 5, 9, 1, 7, 6, 7, 10, 2, 8, 19, 10, 3, 4, 1, 8, 7, 6, 3, 11, 5, 13, 8, 5, 6, 7, 3, 5, 7, 10, 5, 9, 4, 5, 5, 7, 4, 2, 10, 5, 17, 5, 10, 5, 12, 4, 8, 14, 14, 8, 5, 3, 6, 10, 8, 10, 7, 12, 12, 8, 8, 14, 7, 10, 12, 9, 5, 14, 3, 5, 10, 8, 8, 7, 9, 9, 6, 5, 10, 10, 6, 11, 7, 7, 7, 8, 7, 10, 14, 4, 4, 3, 5, 9, 4, 10, 8, 9	743	
1 Complement-deficient	3, 4, 1, 0, 3, 1, 1, 5, 1, 2, 3, 4, 0, 0, 6, 2, 3, 0, 0, 5, 1, 1, 8, 0, 0, 8, 4, 1, 4, 1, 1, 7, 4, 4, 2, 2, 3, 0, 1, 2, 1, 4, 7, 1, 6, 0, 5, 4, 0, 1, 6, 1, 0, 2, 1, 0, 4, 0, 0, 0, 3, 0, 1, 0, 2, 6, 0, 2, 0, 0, 2, 7, 3, 2, 5, 1, 3, 6, 2, 8, 0, 2, 1, 8, 0, 2, 3, 0, 6, 0, 0, 2, 2, 7, 3, 2, 3, 2, 3, 0	240	0.323
2 Normal	18, 8, 0, 4, 6, 1, 6, 5, 8, 3, 18, 2, 0, 0, 15, 8, 0, 6, 4, 6, 9, 10, 10, 5, 6, 7, 9, 14, 9, 8, 11, 7, 6, 6, 5, 10, 7, 10, 3, 4, 9, 12, 14, 10, 8, 12, 9, 6, 7, 9, 3, 8, 15, 19, 6, 6, 10, 0, 6, 8, 7, 14, 12, 12, 5, 9, 25, 10, 10, 5, 3, 17, 14, 4, 16, 8, 2, 12, 4, 12, 9, 9, 14, 16, 8, 6, 5, 10, 6, 18, 18, 9, 7, 12, 15, 5, 20, 10, 18, 10	877	
2 Complement-deficient	9, 4, 3, 11, 8, 0, 2, 6, 8, 6, 4, 5, 0, 0, 0, 3, 4, 0, 5, 6, 0, 0, 5, 0, 0, 2, 6, 8, 0, 5, 9, 10, 2, 0, 0, 4, 2, 4, 3, 5, 8, 2, 12, 6, 2, 0, 2, 1, 2, 3, 4, 15, 1, 1, 1, 3, 2, 4, 4, 4, 3, 0, 5, 3, 0, 2, 2, 12, 0, 6, 2, 2, 2, 4, 4, 4, 15, 2, 5, 1, 1, 8, 4, 2, 4, 18, 0, 3, 4, 2, 3, 10, 0, 0, 3, 3, 3, 1, 0	369	0.4207
3 Normal	9, 10, 6, 5, 5, 8, 7, 6, 8, 11, 10, 6, 0, 9, 9, 10, 7, 12, 6, 18, 0, 10, 12, 8, 9, 12, 10, 8, 8, 0, 8, 9, 14, 10, 9, 13, 12, 10, 12, 15, 9, 11, 10, 10, 5, 12, 11, 12, 10, 8, 10, 10, 0, 12, 11, 9, 14, 11, 11, 6, 9, 14, 8, 0, 6, 11, 9, 9, 6, 0, 6, 5, 9, 10, 11, 9, 9, 13, 4, 7, 0, 4, 5, 13, 11, 7, 9, 10, 11, 8, 0, 9, 11, 10, 1, 16, 12, 5, 5, 11	856	
3 Complement-deficient	5, 4, 6, 3, 5, 0, 9, 12, 10, 3, 5, 3, 0, 5, 3, 2, 6, 8, 3, 1, 0, 5, 4, 3, 6, 3, 0, 6, 9, 8, 11, 9, 10, 7, 8, 6, 1, 0, 7, 0, 1, 6, 9, 12, 0, 5, 3, 7, 7, 4, 4, 5, 8, 4, 0, 6, 6, 6, 8, 8, 4, 7, 10, 6, 2, 6, 5, 5, 6, 0, 0, 0, 0, 6, 4, 3, 5, 4, 3, 4, 5, 10, 1, 0, 3, 3, 6, 6, 0, 4, 4, 5, 0, 2, 5, 7, 5, 3, 8, 1	463	0.5408

TABLE 4—*Continued*

GUINEA-PIG		TOTAL	INDEX
4 Normal	1, 5, 4, 3, 4, 2, 7, 6, 3, 0, 8, 3, 8, 7, 9, 10, 6, 11, 0, 12, 11, 12, 1, 2, 10, 10, 8, 1, 1, 2, 0, 8, 1, 10, 7, 5, 7, 8, 3, 5, 6, 11, 10, 5, 10, 2, 2, 5, 3, 4,	279	
4 Complement-deficient	8, 0, 5, 1, 1, 1, 2, 1, 1, 4, 3, 4, 2, 7, 5, 4, 0, 8, 3, 0, 3, 4, 4, 7, 5, 5, 3, 2, 2, 4, 6, 2, 1, 4, 3, 4, 5, 0, 3, 9, 4, 4, 3, 7, 5, 5, 1, 5, 6, 4.	180	0.6451
5 Normal	6, 1, 7, 12, 33, 1, 1, 10, 8, 8, 6, 2, 1, 8, 5, 6, 9, 0, 1, 3, 1, 10, 0, 1, 12, 17, 22, 8, 0, 0, 0, 4, 6, 9, 3, 0, 22, 5, 9, 12, 4, 3, 0, 9, 4, 15, 15, 1, 3, 0, 3, 2, 9, 7, 11, 1, 21, 19, 3, 2, 2, 8, 31, 0, 40, 8, 12, 0, 15, 0, 2, 10, 2, 5, 4, 0, 9, 4, 4, 29, 8, 9, 6, 14, 24, 14, 3, 14, 0, 1, 8, 5, 11, 1, 16, 7, 23, 12, 3, 19.	784	
5 Complement-deficient	2, 0, 8, 0, 7, 0, 25, 0, 0, 5, 0, 30, 0, 0, 5, 0, 15, 0, 2, 0, 1, 0, 0, 0, 8, 0, 6, 6, 0, 23, 29, 0, 0, 0, 1, 0, 6, 1, 29, 16, 0, 0, 32, 7, 8, 6, 0, 0, 0, 1, 0, 0, 0, 3, 2, 0, 2, 0, 0, 2, 2, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 7, 10, 0, 0, 0, 0, 1, 2, 2, 3, 4, 9, 3, 0, 0, 0, 30, 0, 0, 0, 0.	361	0.4604
6* Normal	14, 0, 0, 7, 3, 0, 3, 6, 2, 0, 34, 1, 6, 12, 3, 5, 2, 5, 6, 41, 32, 8, 0, 6, 16, 2, 0, 0, 6, 4, 1, 14, 17, 27, 8, 0, 0, 20, 2, 0, 7, 5, 7, 3, 2, 20, 0, 1, 3, 26.	387	
6* Complement-deficient	10, 0, 0, 0, 0, 1, 8, 10, 0, 0, 2, 20, 0, 0, 3, 5, 0, 2, 0, 15, 1, 25, 0, 8, 15, 0, 2, 1, 0, 0, 0, 0, 0, 1, 0, 7, 0, 4, 25, 0, 1, 1, 34, 0, 0, 0, 0, 0, 26, 2.	229	0.5917
7 Normal	0, 16, 6, 12, 5, 2, 8, 6, 1, 2, 7, 9, 7, 15, 4, 1, 0, 2, 10, 10, 9, 8, 9, 8, 14, 8, 25, 9, 16, 18, 12, 10, 25, 25, 9, 12, 4, 2, 4, 14, 14, 4, 9, 5, 3, 0, 0, 0, 3, 0.	402	
7 Complement-deficient	2, 8, 3, 3, 2, 3, 7, 2, 3, 8, 0, 5, 5, 1, 1, 4, 0, 5, 5, 3, 2, 6, 3, 8, 3, 0, 0, 0, 0, 4, 25, 2, 3, 4, 2, 4, 2, 3, 0, 5, 4, 1, 0, 0, 1, 5, 1, 2, 1, 3.	164	0.4079

* Counted as a check on number 5 by the serologist of the State Laboratory.

TABLE 4—*Continued*

GUINEA-PIG		TOTAL	INDEX
8	Normal	50, 4, 1, 3, 33, 16, 10, 2, 1, 1, 13, 11, 22, 17, 25, 14, 12, 29, 15, 20, 19, 23, 4, 6, 3, 4, 20, 25, 22, 2, 15, 42, 6, 32, 4, 30, 4, 2, 0, 8, 8, 1, 15, 6, 12, 0, 0, 19, 12, 10	653	
	Complement-deficient	1, 0, 0, 0, 0, 49, 0, 3, 3, 30, 0, 0, 3, 2, 0, 2, 14, 32, 0, 0, 25, 1, 1, 38, 0, 0, 11, 4, 5, 5, 2, 0, 4, 7, 50, 0, 12, 7, 0, 2, 20, 1, 4, 0, 0, 0, 0, 2, 4, 10	354	0.5421
9	Normal	25, 1, 6, 32, 6, 28, 5, 16, 30, 6, 4, 24, 12, 7, 9, 4, 10, 18, 12, 37, 10, 13, 29, 3, 7, 10, 3, 3, 4, 5, 16, 43, 4, 2, 5, 11, 40, 5, 11, 0, 0, 0, 14, 3, 0, 0, 0, 12, 2, 4, 0, 0, 5, 8, 4, 7, 0,3, 1, 14, 9, 7, 0, 0, 0, 7, 4, 1, 0, 0, 8, 8, 30, 17, 1, 6, 5, 0, 8, 1, 5, 28, 6, 0, 15, 15, 1, 2, 0, 19, 12, 6, 0, 36, 8, 11, 2, 17, 6, 8	902	
	Complement-deficient	24, 5, 0, 0, 0, 0, 0, 0, 0, 0, 0, 4, 0, 0, 0, 0, 23, 0, 0, 0, 0, 0, 4, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 4, 0, 0, 0, 0, 0, 40, 0, 10, 12, 6, 5, 0, 0, 0, 4, 40, 38, 0, 3, 40, 3, 1, 0, 6, 20, 0, 0, 4, 4, 0, 2, 0, 0, 20, 0, 0, 0, 2, 2, 1, 2, 1, 2, 3, 0, 0, 24, 0, 0, 0, 20, 0, 4, 6, 8, 0, 0, 0, 40, 2, 1, 0, 0, 1, 2, 4	447	0.4955
10	Normal	60, 0, 0, 16, 49, 2, 6, 39, 1, 12, 35, 40, 0, 2, 4, 7, 2, 0, 0, 10, 2, 0, 4, 9, 4, 1, 0, 4, 50, 0, 8, 15, 3, 12, 2, 8, 4, 26, 6, 4, 0, 6, 2, 3, 0, 38, 0, 8, 3, 9, 0, 0, 29, 7, 19, 4, 3, 26, 4, 0, 5, 10, 3, 3, 2, 15, 1, 53, 3, 4, 10, 23, 1, 0, 0, 57, 5, 30, 37, 28, 42, 0, 0, 4, 31, 33, 0, 41, 0, 0, 5, 7, 10, 7, 26, 9, 24, 2, 15, 1	1155	
	Complement-deficient	56, 0, 12, 0, 29, 49, 34, 0, 0, 0, 53, 6, 36, 0, 6, 0, 6, 26, 11, 17, 0, 11, 10, 3, 19, 2, 0, 3, 22, 0, 5, 2, 28, 5, 0, 2, 0, 10, 3, 0, 3, 2, 0, 0, 5, 3, 0, 4, 0, 6, 6, 0, 0, 6, 1, 0, 5, 9, 0, 17, 0, 2, 10, 0, 3, 0, 6, 3, 0, 0, 34, 1, 3, 0, 0, 3, 0, 3, 12, 0, 6, 0, 3, 5, 6, 0, 0, 0, 52, 3, 0, 39, 0, 0, 15, 36, 0, 3, 0, 9	799	0.6839

TABLE 4—*Continued*

GUINEA-PIG		TOTAL	INDEX
11*	Normal..... 55, 7, 45, 10, 10, 0, 27, 0, 2, 2, 0, 15, 3, 7, 10, 13, 2, 13, 0, 4, 7, 13, 58, 18, 5, 0, 4, 17, 3, 15, 0, 12, 60, 0, 5, 5, 3, 3, 32, 10, 40, 0, 5, 0, 3, 0, 9, 10, 0, 5.....	587	
	Complement-deficient... 50, 0, 24, 0, 0, 0, 0, 2, 2, 4, 42, 10, 2, 20, 5, 25, 8, 3, 6, 0, 2, 34, 6, 10, 1, 2, 0, 2, 18, 0, 0, 2, 2, 0, 2, 2, 4, 30, 2, 2, 0, 32, 7, 20, 3, 2, 3, 24, 18, 6.....	439	0.7461
12	Normal..... 10, 0, 20, 4, 12, 2, 2, 20, 0, 0, 0, 0, 3, 2, 2, 0, 0, 0, 0, 23, 1, 18, 14, 0, 0, 0, 0, 0, 0, 0, 0, 2, 0, 2, 0, 12, 0, 5, 2, 20, 0, 0, 2, 5, 5, 5, 11, 37, 9, 0, 0, 0, 0, 0, 0, 0, 0, 14, 0, 0, 0, 0, 0, 0, 46, 0, 0, 0, 0, 1, 30, 25, 40, 25, 0, 37, 25, 0, 0, 31, 12, 0, 0, 3, 0, 0, 0, 0, 0, 0, 57, 0, 26, 0, 0, 0, 0, 12, 50, 40, 11.....	735	
	Complement-deficient..... 5, 0, 0, 1, 0, 2, 2, 10, 0, 2, 3, 1, 0, 7, 4, 0, 2, 0, 25, 4, 2, 0, 0, 10, 10, 0, 0, 2, 0, 0, 0, 0, 0, 9, 0, 0, 6, 0, 0, 0, 5, 0, 2, 0, 3, 8, 1, 7, 6, 0, 0, 0, 7, 15, 32, 2, 2, 0, 2, 0, 0, 15, 5, 11, 0, 16, 0, 0, 0, 5, 6, 0, 3, 2, 0, 12, 2, 0, 0, 5, 13, 0, 0, 0, 0, 3, 8, 2, 13, 0, 2, 1, 0, 0, 17, 0, 0, 10, 0, 0.....	350	0.4761
13	Normal..... 21, 0, 12, 15, 6, 0, 12, 8, 0, 18, 0, 0, 28, 19, 15, 0, 6, 18, 27, 7, 6, 17, 7, 9, 0, 5, 0, 14, 3, 25, 3, 0, 25, 20, 48, 20, 10, 0, 13, 6, 0, 0, 22, 20, 2, 6, 38, 2, 6, 0, 0, 7, 6, 2, 0, 23, 0, 16, 2, 15, 2, 23, 18, 16, 28, 19, 39, 0, 0, 17, 10, 4, 1, 5, 42, 1, 0, 21, 3, 15, 0, 12, 7, 8, 0, 3, 5, 0, 6, 18, 11, 6, 18, 0, 17, 24, 27, 13, 8, 25....	1082	
	Complement-deficient.... 17, 0, 0, 0, 27, 7, 22, 0, 31, 6, 34, 0, 0, 0, 0, 24, 0, 0, 0, 0, 0, 18, 23, 2, 10, 7, 31, 6, 0, 0, 16, 20, 2, 5, 0, 0, 0, 27, 2, 5, 0, 0, 0, 23, 2, 0, 12, 5, 0, 2, 3, 5, 1, 1, 12, 18, 22, 0, 0, 0, 0, 4, 0, 0, 0, 0, 18, 0, 22, 0, 19, 0, 0, 9, 0, 6, 0, 45, 0, 0, 4, 0, 24, 0, 0, 14, 18, 18, 4, 0, 20, 0, 0, 0, 16, 5, 0, 0, 14, 13.....	721	0.6663

* Counted as a check on number 10 by the serologist of the State Laboratory.

TABLE 4—*Continued*

GUINEA-PIG		TOTAL	INDEX
14	Normal	1, 2, 6, 4, 0, 17, 2, 3, 21, 12, 5, 0, 0, 6, 0, 22, 0, 5, 4, 0, 0, 3, 10, 0, 0, 4, 2, 11, 1, 6, 2, 7, 6, 8, 0, 6, 14, 15, 2, 0, 12, 12, 2, 0, 2, 0, 7, 13, 4, 2, 1, 8, 1, 0, 12, 3, 8, 5, 15, 5, 1, 9, 8, 2, 5, 1, 26, 12, 3, 2, 2, 36, 4, 29, 0, 2, 5, 37, 5, 2, 1, 4, 2, 2, 4, 20, 6, 1, 0, 5, 4, 2, 14, 0, 15, 0, 27, 2, 4, 12	635	
	Complement-deficient	11, 0, 0, 1, 0, 0, 11, 0, 0, 0, 0, 17, 0, 3, 0, 22, 6, 0, 5, 0, 0, 4, 3, 0, 2, 2, 0, 1, 0, 0, 0, 0, 2, 1, 20, 2, 0, 0, 0, 0, 0, 0, 0, 0, 8, 4, 13, 6, 14, 2, 0, 0, 15, 2, 7, 0, 1, 0, 0, 0, 0, 2, 31, 0, 0, 0, 0, 0, 1, 0, 0, 0, 7, 0, 0, 17, 0, 6, 1, 1, 0, 2, 1, 8, 1, 4, 1, 4, 0, 1, 0, 0, 4, 0, 10, 0, 0, 0, 1, 10	298	0.4551
15	Normal	4, 0, 7, 17, 13, 0, 30, 7, 8, 15, 11, 7, 12, 0, 0, 9, 10, 3, 4, 0, 8, 3, 0, 17, 8, 0, 9, 10, 18, 2, 5, 2, 1, 17, 0, 0, 1, 7, 11, 0, 0, 2, 7, 6, 2, 9, 0, 4, 18, 1, 0, 0, 15, 9, 14, 6, 2, 0, 26, 17, 17, 0, 0, 5, 11, 3, 31, 3, 1, 2, 26, 5, 13, 27, 14, 8, 9, 9, 33, 15, 5, 9, 3, 15, 11, 19, 18, 4, 2, 0, 1, 0, 11, 2, 4, 6, 2, 8, 10, 9	785	
	Complement-deficient	10, 0, 0, 10, 3, 0, 1, 0, 21, 16, 23, 11, 0, 0, 8, 8, 2, 2, 0, 3, 3, 27, 7, 0, 17, 0, 5, 16, 6, 6, 4, 0, 0, 18, 6, 0, 8, 2, 31, 1, 18, 1, 0, 2, 0, 4, 17, 1, 0, 0, 8, 8, 4, 8, 5, 1, 0, 3, 4, 4, 0, 0, 0, 0, 1, 7, 3, 2, 2, 6, 2, 4, 2, 8, 0, 0, 3, 2, 8, 13, 4, 1, 7, 10, 0, 5, 15, 4, 5, 0, 2, 0, 2, 18, 6, 1, 7, 6, 13, 0	532	0.6777
16	Normal	1, 0, 7, 25, 13, 4, 14, 0, 4, 14, 50, 1, 10, 0, 25, 0, 50, 0, 0, 2, 23, 2, 2, 5, 7, 6, 36, 0, 0, 0, 0, 1, 2, 9, 14, 4, 4, 6, 26, 11, 32, 0, 0, 5, 8, 31, 25, 10, 8, 37, 0, 9, 17, 14, 0, 0, 0, 4, 15, 17, 6, 13, 4, 0, 12, 20, 2, 8, 0, 23, 0, 15, 2, 24, 13, 4, 12, 4, 14, 19, 0, 0, 20, 25, 0, 0, 12, 0, 12, 0, 21, 10, 7, 23, 0, 8, 1, 0, 0, 9	953	
	Complement-deficient	1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 9, 7, 8, 0, 40, 2, 2, 0, 2, 0, 0, 0, 0, 5, 20, 0, 0, 0, 17, 5, 3, 26, 2, 2, 2, 0, 0, 0, 0, 0, 1, 24, 3, 7, 5, 0, 0, 0, 0, 9, 0, 0, 3, 8, 10, 0, 0, 0, 0, 0, 0, 0, 0, 9, 2, 0, 0, 12, 30, 1, 10, 4, 0, 0, 3, 11, 0, 0, 0, 0, 0, 0, 0, 5, 0, 11, 0, 3, 2, 0, 0, 2, 0, 0, 0, 27, 19	374	0.2548

TABLE 4—*Continued*

GUINEA-PIG		TOTAL	INDEX
17	Normal	8, 13, 7, 5, 7, 0, 0, 0, 8, 1, 11, 26, 7, 1, 2, 6, 7, 11, 8, 4, 4, 4, 2, 17, 0, 7, 4, 10, 6, 10, 16, 1, 1, 0, 4, 6, 1, 2, 8, 5, 5, 4, 10, 8, 11, 12, 4, 3, 4, 0, 2, 5, 31, 7, 0, 8, 9, 1, 10, 2, 5, 4, 4, 4, 4, 8, 7, 6, 8, 9, 3, 0, 2, 1, 8, 10, 8, 27, 3, 4, 6, 11, 0, 1, 5, 4, 8, 0, 3, 2, 5, 7, 4, 0, 2, 4, 0, 0, 6, 2	571	0.3765
	Complement-deficient	1, 0, 0, 0, 0, 2, 2, 10, 0, 0, 2, 0, 10, 8, 0, 6, 2, 8, 1, 0, 8, 6, 9, 0, 3, 0, 0, 0, 0, 2, 2, 3, 0, 1, 2, 5, 4, 5, 0, 6, 0, 0, 0, 1, 0, 2, 4, 0, 0, 0, 0, 0, 5, 4, 2, 1, 7, 0. 8, 1, 5, 7, 1, 1, 0, 0, 2, 1, 2, 0, 4, 0, 0, 1, 4, 1, 0, 0, 5, 0, 4, 0, 0, 0, 0, 5, 0, 5, 2, 6, 0, 0, 6, 0, 2, 4, 4, 0, 0, 0	215	
18*	Normal	6, 4, 4, 3, 4, 4, 6, 7, 11, 6, 2, 4, 4, 0, 9, 5, 10, 2, 6, 4, 6, 4, 3, 3, 7, 15, 1, 6, 6, 0, 12, 5, 7, 2, 15, 4, 12, 5, 2, 9, 6, 8, 0, 3, 2, 7, 13, 12, 2, 10	288	0.3368
	Complement-deficient	4, 0, 0, 3, 1, 2, 2, 3, 0, 0, 5, 0, 0, 2, 0, 4, 0, 2, 7, 0, 11, 0, 6, 0, 8, 0, 0, 0, 0, 0, 0, 2, 0, 0, 3, 0, 0, 0, 2, 0, 10, 1, 5, 3, 0, 0, 5, 3, 0, 3	97	
19	Normal	1, 0, 0, 7, 0, 0, 6, 3, 0, 6, 3, 5, 2, 5, 9, 3, 1, 1, 2, 6, 2, 0, 7, 1, 2, 14, 4, 0, 0, 4, 4, 6, 6, 6, 5, 5, 5, 4, 0, 6, 6, 3, 0, 8, 2, 10, 2, 4, 5, 1, 2, 6, 3, 6, 2, 0, 0, 3, 2, 22, 12, 16, 5, 8, 6, 3, 0, 1, 3, 4, 6, 16, 9, 0, 7, 1, 12, 0, 10, 3, 2, 4, 0, 0, 0, 4, 0, 6, 9, 6, 7, 6, 0, 2, 1, 0, 0, 6, 9, 14	426	0.4976
	Complement-deficient	7, 0, 9, 12, 0, 0, 16, 16, 1, 1, 11, 0, 0, 7, 0, 0, 4, 6, 0, 0, 0, 0, 0, 7, 0, 0, 11, 2, 5, 0, 0, 0, 3, 0, 0, 0, 1, 2, 2, 0, 0, 0, 5, 0, 5, 0, 2, 0, 0, 2, 3, 0, 0, 0, 0, 6, 0, 2, 0, 1, 5, 0, 9, 0, 5, 0, 0, 3, 0, 0, 8, 0, 2, 0, 0, 0, 0, 0, 0, 4, 6, 0, 2, 0, 0, 0, 3, 0, 0, 5, 1, 0, 0, 0, 0, 1, 3, 5, 1, 0, 0	212	

* Counted as a check on number 17 by the agricultural bacteriologist.

TABLE 4—*Continued*

GUINEA-PIG			TOTAL	INDEX
20	Normal	8, 4, 4, 2, 5, 3, 6, 3, 1, 5, 0, 2, 0, 20, 2, 3, 7, 4, 15, 6, 0, 1, 6, 5, 3, 2, 7, 6, 19, 2, 7, 12, 6, 4, 12, 11, 9, 1, 2, 14, 18, 5, 0, 5, 0, 9, 0, 5, 5, 14, 1, 0, 9, 0, 2, 4, 6, 1, 19, 8, 2, 4, 0, 0, 14, 13, 11, 8, 8, 7, 10, 8, 14, 3, 18, 1, 10, 12, 7, 0, 5, 7, 10, 7, 6, 0, 5, 4, 1, 4, 8, 6, 2, 8, 6, 5, 2, 0, 13, 3	592	
	Complement-deficient	3, 0, 0, 2, 0, 6, 0, 3, 0, 0, 8, 2, 0, 0, 0, 0, 0, 0, 1, 6, 0, 0, 0, 0, 0, 1, 0, 0, 1, 0, 0, 4, 0, 0, 0, 2, 0, 1, 0, 2, 0, 3, 0, 6, 0, 2, 0, 0, 3, 1, 2, 1, 2, 5, 0, 9, 0, 3, 2, 0, 1, 0, 21, 0, 6, 0, 0, 0, 0, 3, 5, 0, 9, 0, 0, 0, 0, 0, 0, 0, 0, 0, 3, 4, 4, 0, 1, 0, 0, 0, 0, 0, 5, 4, 0, 6, 0, 2, 0, 0,	155	0.2618
21	Normal	3, 16, 2, 4, 3, 4, 13, 0, 6, 6, 38, 27, 23, 23, 7, 7, 3, 14, 9, 2, 6, 0, 6, 6, 6, 18, 2, 5, 6, 2, 10, 5, 6, 4, 1, 10, 10, 3, 4, 10, 3, 4, 13, 2, 1, 1, 5, 3, 15, 0, 1, 1, 6, 0, 6, 0, 1, 6, 12, 6, 9, 12, 12, 24, 2, 3, 8, 10, 8, 13, 4, 0, 11, 12, 9, 4, 0, 2, 0, 13, 0, 20, 18, 5, 8, 2, 3, 5, 4, 2, 7, 11, 2, 15, 0, 3, 5, 2, 12, 8	704	
	Complement-deficient	7, 13, 6, 0, 10, 1, 1, 0, 10, 4, 2, 10, 2, 2, 4, 6, 5, 1, 13, 0, 5, 0, 4, 10, 15, 1, 0, 3, 18, 3, 0, 0, 3, 4, 0, 3, 8, 9, 20, 0, 0, 0, 2, 4, 3, 5, 6, 0, 1, 5, 0, 7, 8, 0, 0, 10, 2, 4, 7, 0, 6, 5, 0, 0, 0, 11, 0, 0, 2, 4, 0, 8, 2, 2, 6, 4, 10, 0, 4, 4, 0, 0, 3, 0, 0, 0, 4, 6, 12, 6, 0, 8, 0, 20, 0, 9, 20, 8, 10, 0	441	0.6264
22	Normal	1, 3, 8, 0, 0, 11, 0, 1, 12, 0, 1, 9, 2, 24, 3, 5, 4, 1, 11, 0, 4, 10, 2, 14, 6, 1, 0, 3, 13, 11, 2, 3, 12, 6, 1, 12, 4, 8, 7, 11, 2, 26, 2, 17, 1, 1, 2, 0, 6, 10, 21, 0, 6, 14, 1, 7, 2, 0, 2, 6, 0, 5, 2, 3, 3, 3, 5, 1, 6, 1, 6, 4, 3, 1, 21, 6, 0, 7, 4, 0, 6, 8, 28, 8, 14, 0, 15, 13, 8, 0, 5, 6, 0, 9, 40, 4, 8, 5, 15, 3	628	
	Complement-deficient	10, 6, 0, 9, 17, 2, 2, 0, 8, 0, 0, 1, 0, 5, 2, 1, 9, 0, 2, 2, 0, 4, 0, 0, 5, 0, 0, 0, 6, 0, 2, 0, 0, 34, 21, 18, 0, 7, 2, 16, 9, 0, 0, 4, 6, 8, 0, 0, 6, 3, 6, 0, 0, 20, 0, 0, 0, 0, 2, 13, 17, 10, 0, 7, 0, 6, 0, 0, 4, 0, 6, 0, 2, 2, 1, 10, 0, 3, 4, 12, 0, 0, 17, 0, 2, 3, 25, 2, 25, 2, 0, 0, 5, 0, 0, 0, 0, 0, 0, 1	434	0.6910

TABLE 4—*Continued*

GUINEA-PIG		TOTAL	INDEX
23 Normal	14, 6, 2, 3, 0, 1, 0, 2, 0, 6, 1, 14, 13, 6, 11, 12, 7, 5, 11, 0, 5, 0, 0, 17, 4, 10, 9, 6, 4, 0, 5, 7, 0, 4, 8, 7, 10, 1, 1, 11, 0, 2, 12, 6, 20, 1, 16, 3, 12, 25, 10, 0, 6, 10, 13, 2, 15, 5, 5, 5, 0, 0, 10, 4, 0, 14, 2, 9, 10, 5, 5, 0, 10, 3, 3, 1, 5, 0, 2, 12, 5, 10, 2, 3, 6, 6, 2, 12, 0, 0, 17, 2, 16, 1, 11, 3, 10, 1, 14, 0.....	607	
23 Complement-deficient	2, 0, 0, 0, 5, 2, 3, 1, 6, 3, 0, 0, 2, 4, 1, 4, 0, 0, 0, 1, 0, 0, 5, 1, 0, 0, 4, 2, 3, 9, 0, 5, 3, 0, 2, 0, 4, 0, 2, 6, 2, 0, 0, 0, 4, 0, 0, 0, 0, 0, 8, 4, 0, 2, 0, 2, 3, 1, 4, 0, 0, 2, 4, 2, 6, 5, 5, 4, 3, 2, 0, 0, 3, 0, 3, 0, 0, 2, 1, 1, 4, 0, 2, 1, 3, 0, 0, 0, 1, 5, 2, 0, 2, 0, 0, 1, 0, 0, 2, 1.....	177	0.2915
24 Normal	4, 7, 22, 1, 2, 0, 5, 9, 10, 0, 0, 0, 2, 0, 9, 13, 6, 0, 2, 2, 9, 11, 44, 5, 3, 3, 22, 3, 1, 4, 0, 5, 1, 0, 0, 22, 0, 8, 16, 4, 7, 2, 2, 2, 3, 2, 5, 0, 5, 3, 2, 6, 3, 4, 5, 10, 7, 0, 3, 5, 22, 4, 2, 27, 23, 14, 18, 0, 0, 8, 5, 20, 3, 5, 5, 8, 0, 2, 14, 38, 14, 14, 7, 13, 6, 9, 12, 6, 0, 5, 7, 2, 15, 10, 14, 6, 8, 20, 2, 20	739	
24 Complement-deficient	4, 4, 1, 2, 4, 2, 0, 0, 0, 1, 0, 0, 0, 1, 0, 3, 5, 1, 0, 0, 0, 0, 0, 0, 1, 0, 0, 0, 0, 0, 4, 0, 0, 0, 0, 0, 3, 6, 0, 5, 5, 2, 0, 14, 0, 2, 0, 0, 5, 0, 0, 0, 0, 0, 0, 0, 0, 2, 0, 0, 0, 3, 2, 0, 2, 2, 1, 0, 1, 0, 3, 2, 0, 0, 5, 0, 0, 0, 0, 0, 0, 0, 0, 7, 6, 0, 0, 1, 7, 0, 0, 8, 6, 16, 2, 0, 7, 3, 4, 2, 0....	165	0.2232
25 Normal	8, 1, 0, 12, 1, 2, 3, 0, 1, 1, 2, 9, 4, 2, 0, 12, 2, 6, 0, 0, 18, 20, 2, 2, 0, 27, 4, 0, 2, 0, 1, 3, 3, 1, 16, 9, 18, 5, 7, 5, 6, 2, 3, 0, 0, 1, 0, 0, 9, 4, 2, 11, 11, 1, 1, 0, 2, 12, 9, 0, 15, 4, 19, 9, 11, 7, 2, 25, 3, 2, 5, 12, 15, 0, 5, 4, 1, 1, 2, 0, 2, 4, 33, 3, 6, 3, 1, 5, 2, 10, 5, 0, 3, 0, 10, 1, 26, 2, 2, 4.....	547	
25 Complement-deficient	6, 0, 4, 15, 0, 0, 7, 4, 2, 0, 0, 0, 6, 0, 0, 19, 0, 0, 0, 6, 6, 5, 10, 5, 5, 0, 0, 0, 7, 0, 0, 4, 4, 0, 0, 0, 4, 0, 3, 4, 2, 2, 5, 5, 2, 0, 3, 0, 0, 2, 5, 0, 2, 0, 3, 0, 1, 0, 0, 1, 0, 2, 0, 0, 0, 5, 5, 0, 0, 12, 0, 0, 0, 8, 2, 0. 0, 0, 0, 0, 4, 9, 5, 2, 0, 0, 0, 0, 11, 0, 6, 0, 0, 5, 21, 0, 12, 2, 0, 2, 6	272	0.4936

TABLE 4—*Continued*

GUINEA-PIG			TOTAL	INDEX
26	Normal	9, 9, 25, 36, 4, 4, 2, 0, 0, 2, 20, 2, 3, 15, 0, 0, 7, 4, 6, 8, 3, 0, 2, 0, 0, 3, 3, 3, 3, 6, 4, 7, 43, 3, 2, 30, 3, 1, 21, 0, 0, 8, 29, 2, 0, 1, 0, 4, 15, 9, 4, 2, 18, 6, 0, 6, 0, 0, 3, 4, 2, 4, 10, 20, 0, 2, 0, 10, 0, 12, 4, 1, 2, 2, 4, 4, 2, 0, 0, 2, 5, 0, 3, 1, 9, 0, 1, 10, 1, 4, 0, 6, 6, 6, 1, 6, 0, 3, 3, 0, 6.....	553	
	Complement-deficient	1, 0, 0, 3, 2, 0, 4, 1, 0, 3, 6, 2, 0, 1, 9, 0, 0, 2, 18, 0, 0, 24, 0, 33, 3, 7, 4, 1, 3, 0, 3, 10, 2, 2, 0, 2, 4, 2, 3, 0, 0, 0, 2, 3, 20, 0, 8, 6, 4, 7, 8, 0, 0, 4, 7, 3, 1, 5, 0, 0, 4, 6, 4, 4, 0, 0, 0, 1, 0, 0, 0, 1, 0, 2, 5, 0, 0, 9, 0, 0, 4, 2, 0, 2, 0, 18, 0, 0, 6, 2, 0, 8, 26, 3, 0, 13, 0, 0, 7, 0,.....	360	0.6509
27	Normal	2, 0, 4, 8, 0, 2, 0, 6, 4, 2, 14, 11, 6, 0, 3, 5, 0, 3, 0, 10, 7, 4, 21, 20, 1, 17, 4, 38, 40, 23, 1, 3, 4, 7, 2, 5, 10, 7, 0, 8, 1, 14, 5, 0, 38, 11, 3, 2, 0, 0, 2, 21, 0, 12, 5, 8, 0, 1, 5, 3, 3, 2, 20, 18, 13, 0, 2, 4, 0, 4, 6, 30, 3, 4, 18, 3, 13, 6, 5, 3, 0, 14, 2, 5, 0, 8, 6, 17, 11, 18, 36, 2, 6, 0, 0, 0, 3, 0, 13, 5.....	736	
	Complement-deficient	2, 0, 0, 6, 0, 0, 0, 0, 0, 0, 4, 6, 0, 0, 6, 0, 2, 6, 2, 4, 5, 2, 1, 0, 0, 1, 0, 3, 0, 4, 0, 6, 2, 5, 4, 4, 0, 10, 2, 0, 8, 1, 0, 0, 5, 2, 2, 1, 2, 0, 1, 2, 0, 0, 0, 2, 0, 7, 0, 6, 0, 0, 5, 0, 0, 2, 10, 0, 0, 0, 0, 0, 5, 5, 0, 0, 0, 10, 0, 0, 0, 2, 4, 2, 0, 10, 0, 0, 6, 2, 2, 2, 4, 2, 3, 0, 4, 0, 0, 1.....	207	0.3555

NOTE: Each number represents the number of bacteria ingested by one leucocyte.

In the estimation of the average index, which is given at the end of the table, the counts of number 10 are purposely omitted.

TABLE 4—*Concluded*

Summary of all the counts excepting number 10 and its check number 11

	COMPLEMENT-DEFICIENT	NORMAL
	240	743
	369	877
	463	856
	180	279
	361	734
	229	387
	164	402
	354	653
	447	902
	350	735
	721	1,082
	298	635
	532	785
	374	953
	215	571
	97	288
	212	426
	155	592
	441	704
	434	628
	177	607
	165	739
	272	547
	360	553
	207	736
Total	7,817	16,464
Average phagocytic index	3.1268	6.5856
Average opsonic index of complement-deficient serum	0.4748	

The foregoing determinations demonstrate a distinctly subnormal opsonic power in all of the sera of the complement-deficient guinea-pigs. While this result might be thought to indicate some relationship between the opsonic and the complementary functions of serum as has been assumed by some, it would seem to contradict an assumption of identity of those two functions; because in these sera some opsonic property, nearly half, is retained, whereas complementary activity seems to be completely absent (less than 1 per cent of the normal activity).

SUMMARY

The great deficiency noted by Ramon C. Downing in the complementary power of the blood serum of some guinea-pigs, has been confirmed by the writer's experiments.

Complement titrations made by the writer, in which positive and negative control was employed, seemed to indicate that the apparent lack of complement in the blood serum of some guinea-pigs is not due to the presence therein of anything interfering with the action of amboceptor.

The biological tests applied to the blood serum of complement-deficient guinea-pigs which had been systematically immunized by repeated inoculations, gave positive evidence of the presence of both bacteriolytic and agglutinating antibodies; moreover, when inoculated with large doses of live virulent culture, such guinea-pigs manifested more or less active immunity to the bacteria injected.

Titration of the blood serum of complement-deficient guinea-pigs, before and after the production of active immunity, gave practically the same complement titer.

The results of experimental inoculation of one hundred non-immunized complement-deficient guinea-pigs and a like number of complement-normal guinea-pigs, seem to indicate that complement bears some relation to natural immunity.

The results of counting the bacteria in 4800 leucocytes of complement-deficient and complement-normal guinea-pigs show that the phagocytic index of the serum of complement-deficient guinea-pigs is about one half that of the complement-normal animals.

CONCLUSIONS

The conclusions drawn from the foregoing study are as follows:

1. That the apparent lack of complement in the blood of the guinea-pigs used in this work was not caused by inhibition of the amboceptor.

2. That the lack of complement is not necessarily inimical to the production of acquired immunity.

3. That the animal tissues do not make up any deficiency in the complement content of the blood during active immunization.

4. That the complement titer varies with the opsonic-index and in the same direction.

5. That the opsonic determination is too tedious to lend itself readily to practical use.

6. That complement and opsonins bear a definite relation to each other.

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